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# MICROBIC DISSOCIATION IN STREPTOCOCCI<sup>1</sup>

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With the accumulation of a considerable literature which bears either directly or indirectly on microbic dissociation and on bacteriophagic phenomena, one searches with little reward for reports of work on either subject relating to the streptococci. There is perhaps no other bacterial group concerning which so much conflicting information is available. Notoriously difficult to classify even by resort to serologic methods, and even more difficult to lyse with the bacteriophage, there is little wonder that investigators have confined their efforts to those groups of bacteria which afforded a more stable foundation upon which to construct experimental procedures.

In regard to bacterial dissociation Cowan (1922) has probably given us the most concise account of findings concerning the separation of strains of streptococci into rough and smooth types, with a description of the appearance of the colonies. She has also correlated the relative lack of virulence in the R types, which is in line with so much of the work that has been done on other bacterial species. Hadley (1927) has recently reported that Faith Hadley has observed R dissociates giving rise to daughter colonies and has associated spontaneous agglutination with the R types. He also points out the work of Enderlein and that of Charrin and Rogers in relation to changes of virulence of streptococci.

In relation to the bacteriophage there is also little to be found concerning the streptococci. A few reports have appeared detailing the transmissible lysis of streptococci but little else has appeared. My own work (1926a, 1926b, 1927) in this connection was an attempt to explain certain observed facts in

<sup>1</sup> Presented before the Society of American Bacteriologists, December 29, 1927.

the light of the bacteriophage and of d'Herelle's theories concerning its behavior. A critical analysis of the data presented however, leaves us considerably in doubt as Hadley has pointed out as to the relative parts played by the bacteriophage and by that group of phenomena we are pleased to include under the term bacterial dissociation, in the experimental and observational results.

To establish a base from which to work it seems necessary that some detailed observational data be collected concerning bacterial dissociation among streptococci. With this purpose in mind a great many strains of streptococci have been surveyed and a few have been subjected to a very close scrutiny.

The outstanding fact that has been disclosed by this series of observations is the almost universal tendency on the part of streptococci to show dissociating forms when grown on an alkaline (pH 7.8 to 8.0) medium. Some of the strains that are essentially of one type on initial observation always, at some time in their cultural history show colonies of other types. Many of the strains have such a tendency to dissociation that any medium is suitable for the demonstration of the appearance of multiple colony types.

Again one is struck by the extreme variability of the colony types that appear in streptococcus cultures. It is seldom that different strains give colonies that are identical or that the same strain gives identical colonies at different times in its cultural history.

Another striking fact is the frequency with which irregular colonies appear in initial cultures on alkaline agar. As a matter of fact pure smooth type strains are almost never seen when initial cultures furnish the environment for rough dissociation.

These facts indicate the futility of trying to elucidate the mechanism or even the manner of dissociation in streptococci, from the study of a few selected strains. As a foundation for future work in this field many strains have been observed. A succession of colony types that defy placing them in an orderly sequence is the result. Out of the mass however have come

several fairly definite types that may serve as a basis of generalization.

It is obvious that the normal type of streptococcus colony should be the basis for comparison in describing the abnormal types. In the literature the smooth or S type colony is considered normal. If by normal we mean that type which most often occurs, certainly the smooth colony would not qualify in this group. For the purposes of this paper however it is immaterial whether the S type is the "normal" functional one or not. The true S type colony is a small, circular, bluish white, translucent, and homogeneous colony, even under the low power of the microscope. A typical example is pictured in figure 1. This true S type must not be confused with the larger smooth colonies that sometimes occur in dissociating cultures. These confusing colonies are many times the size of the true S colonies, are more opaque, and above all readily give rise to varied dissociation. A colony of this type is pictured in figure 2. Strains of streptococci growing on blood agar or other highly favorable media of a pH near neutrality or slightly acid, show almost 100 per cent, or at least many, S colonies. On an alkaline medium (pH 7.8 to 8.0) I have not yet found a strain that would maintain the true S type colony for many generations. As a matter of fact, one of the serious handicaps to significant experiments with the streptococci in this regard, is the impossibility of maintaining corresponding S and R type strains. If S cultures maintained on a favorable medium so readily give rise to R cultures under the proper conditions there is no assurance that in animal experiments, an inoculated S strain will remain so. Indeed I have observed considerable evidence that dissociation takes place readily *in vivo* among the streptococci, which data will be considered in a forthcoming communication. This instability of the S strains has been particularly unfavorable to experiments with the bacteriophage, since broth suspensions usually undergo rapid dissociation with resulting resistance to the lytic principle and sufficient acidity to inhibit such dissociation, also inhibits bacteriophage action.

Probably the most striking type of a dissociating bacterium is

seen in those cultures generally designated as suicide strains. In a previous publication (Dutton, 1926a) I have described an actively lytic strain of streptococcus which might well serve as an extreme example of a suicide strain. This organism exhibited a lytic tendency on most mediums in a very striking manner. The colonies after fifteen to twenty-four hours were large, watery, smooth and convex. After forty-eight hours they began to flatten and rapidly disappeared from the surface of the medium, leaving no trace of their presence, and never developed secondary colonies. Transplants from these colonies after forty-eight hours were invariably sterile. The behavior of this strain in broth was interesting. If sufficient of a twenty-four-hour agar culture to produce a faint turbidity was washed into a tube of alkaline broth growth rapidly took place until there was a heavy cloud in about 15 hours. At the end of twenty-four to thirty-six hours there remained no trace of suspended organisms, transplants were sterile and no secondary growth took place. It was possible to maintain this culture for about thirty days by daily transplants and the spontaneous lysis of the strain in broth was repeatedly observed. Since then several other strains that have manifested the suicide tendency have come under my observation. None of them have been so striking in their manifestation however. Several have given growth for only one or two transplants and others have grown only in the initial culture. The colony changes were essentially as observed for the strain detailed. Those that have been capable of continued transfer have been so by virtue of the development of secondary colonies that had no tendency to spontaneous lysis. It is possible that the few strains that have given good growth on agar, but failed to give growth in broth even with massive inoculations, are of the same nature. It must be borne in mind that the phenomena of bacterial dissociation are not static but are purely relative in their degree of activity, and that the same fundamental factor may be responsible for manifestations that bear no resemblance in their observable nature.

A second type of dissociating colony, more common than the suicide strains and hardly less striking, is that which shows the

development of secondary colonies. There have been a variety of types of secondary colonies described for other bacterial species. Hadley (1927) has adequately reviewed the literature in this connection. Almost all of the described types have been found in dissociating streptococcus cultures. The most common type is that group which develops in the bare areas of lytic colonies. Morphologically these are S types, and when one is successful in transferring such a secondary colony without contamination from the remaining R cells a typical S culture is obtained. Such an S culture is not stable however, for after several transplants the original, or some other, R type colony makes its appearance and progressively becomes more numerous until the S colonies are lost. Figure 3 illustrates such a lytic colony with small S type secondary colonies in the bare areas.

Another type of colony with secondary colonies is frequently seen. The mother colony does not present a discrete lytic area, but the whole colony becomes flattened and irregular in outline and after several days discrete smooth secondary colonies develop, sometimes in the center of the colony sometimes at the edge. Here again, the transfer cultures from the secondary colonies give rise to S type cultures that are generally not stable. Transfers from the mother colony after the appearance of the secondaries, if care is taken not to carry over any of the secondary growth, are almost invariably sterile. If however, the whole colony is transferred the resulting culture is usually a repetition of the original picture. This is significant in that it indicates that there is some property of the dead cells in the sterile portion of the colony which has the power to stimulate the dissociation of the R type much more rapidly than would take place spontaneously. Filtrates of such a culture do not seem to be as effective in stimulating this dissociation as the substance of the colony itself. Definite evidence has been obtained however, that filtrates of such a growth may under certain circumstances stimulate the appearance of dissociating colonies. Figure 4 illustrates such a colony as that just described.

Still another type of secondary colony bearing growth is that in which the mother colonies do not seem to undergo any appre-



chable change, while the secondary colonies develop as S colonies either on the surface of the growth or in its depth. Often such a colony instead of showing definite secondary colonies will present numerous small irregular "papillae," usually on the surface. I have been unable to secure a transfer uncontaminated with cells of the mother colony from these secondary colonies (fig. 5).

In most of the instances given above, the secondary colonies have been more opaque than the mother colony, although S cultures arising from them have been less opaque than the R strains from which they are developed. In a few instances the secondary colonies have been definitely more transparent,—so much so that except with the low power of the microscope or a good hand lens it was difficult to distinguish them from minute lytic areas.

The changes described above have generally taken place in the course of from three to ten days incubation, either at room temperature or at incubator temperature. Room temperature seems to be slightly more favorable to the dissociation however.

As appears from the illustrations the colonies showing the development of secondary colonies have not been of the true S type. As will be seen later, neither are they extreme R types. For the present we will have to place them in that indefinite intermediate group, which includes a multiplicity of colony types. We have then in these secondary colonies the sudden O-S dissociation.

While the O-S or R-S transformation may take place suddenly through the mechanism of secondary colonies, the opposite, S-R or O-R sudden dissociation has been rarely observed. A few cultures have been studied however, that seem to be in this category. These strains have shown colonies in the intermediate group without the occurrence of S type secondary colonies. Such strains have been difficult to carry in stock cultures because of the rapid death of the cells. An occasional brain agar slant however has been seen to give rise to secondary rough colonies, after a period of from one to two months incubation in the ice box. These R colonies are extremely irregular as to outline, are

opaque and have a granular surface. They are several times as large as the O colonies from which they arise. On transfer, the resulting colony type approaches the original O type and after several transfers reverts entirely to it. It is probable that these colonies represent the extreme R type for streptococci, although it has been impossible to maintain them except by transfers made at long intervals. As a rule there is an interval of varying length between the time that the O colonies become available and the appearance of these extreme R dissociates. I have not yet determined the length of the viability of these colonies, but it is considerably in excess of that of the mother growth. It is necessary that more work be done on this type colony to establish it as the extreme R type. It certainly is of a different order from that of the rough colonies that develop rapidly in streptococcus cultures.

I have never observed the tertiary colonies which Hadley (1927) reports for the *Streptococcus fecalis*. However very few of my observations have been on this particular species.

Still another type of dissociating streptococcus colony which is very striking in its deviation from the S form is the giant colony. These colonies sometime reach a diameter of 5 mm. on plain alkaline agar plates. They are generally flat even in very young cultures, and after several days almost always show secondary S colonies. Sometime they are not so flat and the secondary growth is in the form of irregular papillae. Occasionally no secondary growth is discoverable. Infrequently the end result of the colony is a slow suicide, leaving only a very thin film of almost indistinguishable material on the surface of the agar. In a heavily seeded culture the giant colonies do not develop. In passing it may be noted that the most marked variation of the colonies of whatever type, takes place in thinly planted portions of the cultures. Figure 6 illustrates several types of giant colonies.

The most stable form of R streptococcus colony is perhaps that described by Cowan (1922). This colony is somewhat larger than the S type, irregular in outline, raised but flat, granular and refractile by transmitted light. Many strains eventually stab-

ilize with the great majority of colonies of this type. If stability of a rough colony is to be taken as the criterion of the extreme type, then this colony might well serve as the extreme R. I have been unable to induce S transformation from a growth showing only this type of colony. They never show secondary colonies, lysis, nor any other change within themselves. A few cultures have been encountered that gave 100 per cent colonies of this type on isolation planting, or on the first planting on a medium suitable for detailed observation of the colony form (fig. 13).

A number of cultures planted either from a very old O growth, or from suicide cultures, have given no colonies detectable with the naked eye. Under the low power of the microscope however, numerous very small discrete colonies were seen. Such colonies contain so few cells that it is almost possible to count them even under suitable magnification. They do not seem to undergo lytic changes, but transfers from them are usually sterile. Occasionally such a culture has given rise to an unstable subculture or to an O growth. Such a group of colonies is shown in figure 7.

In certain cultures showing a multiplicity of colony types there have been found a few small, slightly raised, homogeneous, highly transparent colonies that are almost indistinguishable under the microscope. As a rule they can only be detected by the use of oblique lighting. The edges of these colonies are smooth and the outline generally circular. Descriptively, they can be called smooth colonies. They are however decidedly of a different nature from the normal S colonies. Numerous attempts to secure a viable transfer of such colonies have failed. Very occasionally one of these colonies is seen to give rise to an S secondary colony. In that event a viable transplant can be obtained, usually of the micro colony type described in the preceding paragraph, but sometimes of the larger O type. This type of colony is illustrated in figure 8.

Another type of some interest is the so-called fringed colony. These are fairly large, homogeneous, rather opaque, and smooth at the edges, and without secondary colonies. After a varying

period of time a fringe of irregular and granular growth takes place around the outer edge. Rarely secondary colonies develop in this fringe. This picture is sometimes modified by the fringe developing in a small sector only of the colony. Transfers from the body of the colony and the fringe give different types of growths (figs. 9 and 10.)

An immature type of colony has been occasionally seen. Under the microscope it is seen to consist of what is apparently a single chain of cells coiling about over a circumscribed area, forming a network. Sometimes after several days the spaces of this network fill with cells forming a flat colony, of irregular outline, and clearly seen to consist of a single layer of cells (fig. 11). These colonies are unstable, rarely giving growth in transplants. When they do grow, the progeny of these colonies may be either of the several types of rough colonies, but most often the stable type described by Cowan.

A variation of the giant colony is the occurrence of concentric rings of alternate granular and homogeneous growth. This seems likely to be nothing more than the alternate regeneration of S and O growth, or O and R growth (fig. 12). Such colonies are not commonly seen and three or four rings seems to be the limit of their development.

It is seen from these widely varying colony types that dissociation is a very active process in the streptococci. Most of the well defined colony types that have been described in the literature for other species of bacteria are found to occur in this group. At present, I have only an incomplete conception of their sequence, and of factors that govern their appearance. The functional variations accompanying the various colony changes are also obscure. These considerations are under investigation however, with some promise of elucidation. It seems desirable at this time to present the picture of the variations to be expected, as such study may furnish a ground upon which to build more searching inquiries.

#### DISSOCIATION IN A FLUID MEDIUM

In this series of observations only one fluid medium has been extensively used. This is a strong (50 grams Difco dehydrated

veal to 1000 cc. water), veal infusion broth, containing 1 per cent peptone and 0.5 per cent sodium chloride, carried to a reaction of pH 8.0. After sterilization the reaction is usually pH 7.8. In this simple medium practically all strains have been found to give enough growth to work with. The alkalinity is the essential factor in furnishing the proper environment for dissociation. This is easily controlled, and for certain types of experiments offers an ideal arrangement. The reaction is also optimum for bacteriophagic phenomena.

As has been pointed out by other authors, the occurrence of agglutination in fluid mediums is constantly associated with the O or R types. Indeed, the character of the growth in such a broth is the most sensitive indicator yet found of the ability of a seemingly pure S strain to dissociate into the other types. Many strains when first encountered show colonies of such smoothness that it is impossible to determine their exact nature. A culture in broth will determine the true place of the strain. If any agglutination occurs at all in the growth there are sure to develop at some near time in the cultural history of the strain, O or R colonies. If, on the other hand, the culture is definitely homogeneous, it is difficult to secure dissociation in any manner. Alternate agar and broth cultures have proven a ready means to speed up the development of rough forms to S strains. It is significant that the degree of agglutination parallels the degree of roughness of the colonies on agar. Very rough colonies give in broth only a few large clumps of growth, while those that are close to the S type give a very slight agglutination. It has also been demonstrated that certain cultures giving a mixture of S and O types on agar, when put into broth give an agglutinated sediment in an evenly clouded fluid.

It has been found also that the R-S or O-S transformation occurs in broth cultures. Several strains have been observed that showed only O colonies in agar transplants, in the first twenty-four to forty-eight hours incubation, but after several more days, underwent a transformation to the S type and gave only S colonies on transfer to agar. It is probable that this is accomplished by the same mechanism that is at work in the transformation of secondary S colonies on old O or R colonies.

## CELL MORPHOLOGY

While the changes in the colony form among the streptococci are striking, the changes of the cell morphology are no less so. Perhaps the outstanding change observed is the irregularity as to chain formation and the reaction to Gram's stain. The pure S type is constantly Gram-positive and does not form chains. It is rare to see more than two organisms connected in the smooth colonies. The rough dissociated type forms long chains both on agar and in broth, and the reaction to the Gram stain is uncertain. Many of the cells fail to retain the stain at all, while others give an amphophilic reaction. In strains with a strongly lytic activity all the cells are negative. Those portions of the colony that become sterile as detailed above are Gram-negative. Another interesting variation is what I have termed phantom chains. A long chain will have definite gaps in it, in which no cells at all are visible. The cells of the S colonies are uniform in size and shape, while those from the O and R colonies are very irregular in these respects. The most commonly seen variation is the tendency to develop large spherical cells, several times the size of the remaining cells in the colony. That these are not contaminations is apparent from the fact that they develop in the middle of the normal chain. A more detailed consideration of these very interesting cells is to appear in a future paper. A few strains have been seen that give rise to colonies showing a cell morphology almost identical with that of the diphtheroids. In the cases where this has occurred, it was so marked that it strongly indicates a genetic relationship between the streptococci and the diphtheroids.

## RELATION TO VIRULENCE

Cowan (1922) has experimentally demonstrated the relation between virulence and the different growth-types in the streptococci. The S types are the more virulent and the R types relatively avirulent. As a matter of fact, I believe that Cowan's work was done with S and O forms as there was only a relative difference in the virulence of her strains for white mice. I have

observed several R strains that could be given in massive doses to mice without any effect whatever. If the data presented in previous papers is interpreted in the light of dissociation it will be seen that natural spontaneous infections follow this same rule. In those cases that prove fatal the strains are almost invariably S types. They may be unstable on artificial media dissociating into O or R forms in the course of several transfers but on initial isolation they are definitely of the S type. Likewise in those cases in which recovery is spontaneous and rapid the strain is almost sure to be lytic. Infections that present a middle clinical picture show organisms that are R or O types, but without a lytic tendency. This has been so constant that it has been possible to predict the outcome of many infections, especially blood stream infections. Of about forty cases of streptococcus septicemia I have not yet seen death occur if the organism was of the O or R type. As previously reported, it seems that filtrates of lytic strains are of some value therapeutically. In a few cases this has been strikingly so.

#### DISCUSSION

In surveying the data here presented several considerations attract attention. In the first place, one is struck by the almost universal tendency for bacteria of this group, and it appears to be equally true of other groups, to undergo morphologic, cultural, and physiologic variations. Just what will be the ultimate result of this upon the established philosophy of bacteriology, cannot be foreseen. That a radical revision of our conceptions concerning many fundamental things will be necessary, is certain. An understanding of the processes involved in these variations can only follow widespread and painstaking research directed at many species of bacteria, and even at many strains of the same species.

Secondly, it is manifest that an understanding of these variations will clear up many vague aspects of the disease producing capacity of bacteria. It is also likely that upon study of the dissociation of microorganisms in the animal body and the factors which influence it, many of the obscure clinical variations of

infectious diseases will be made clearer. Again that there may be some therapeutic application of these phenomena is more than an idle dream, certainly well worth the earnest effort of research workers.

Thirdly, from a perusal of much in the literature it seems difficult to divorce bacteriophagic activity from microbial dissociation. That the two are related in certain aspects at least, is evident. One may be the precursor of the other, they may both be manifestations of the same factor, or they may be manifestations of the inherent physiologic capacity of the bacterium. Certainly, in a consideration of streptococci the same set of observed data may be interpreted by either d'Herelle's conception of the behavior of the bacteriophage; or by "microbial dissociation." In other groups of organisms this is not so striking, but may be only an indication that the phenomena may vary to a greater degree than is realized at present. Regardless of our ultimate solution of the nature of the bacteriophage, I cannot but feel that the field of bacteriophagic activity must be considerably broadened before much progress is made in elucidating it. Then too, we must cease to regard microbial dissociation as a curious set of cultural variations, but must consider it as deeply significant, and fundamental; with ultimate far reaching effects upon our conception of the nature of bacteria, of infection, and of immunity.

#### SUMMARY

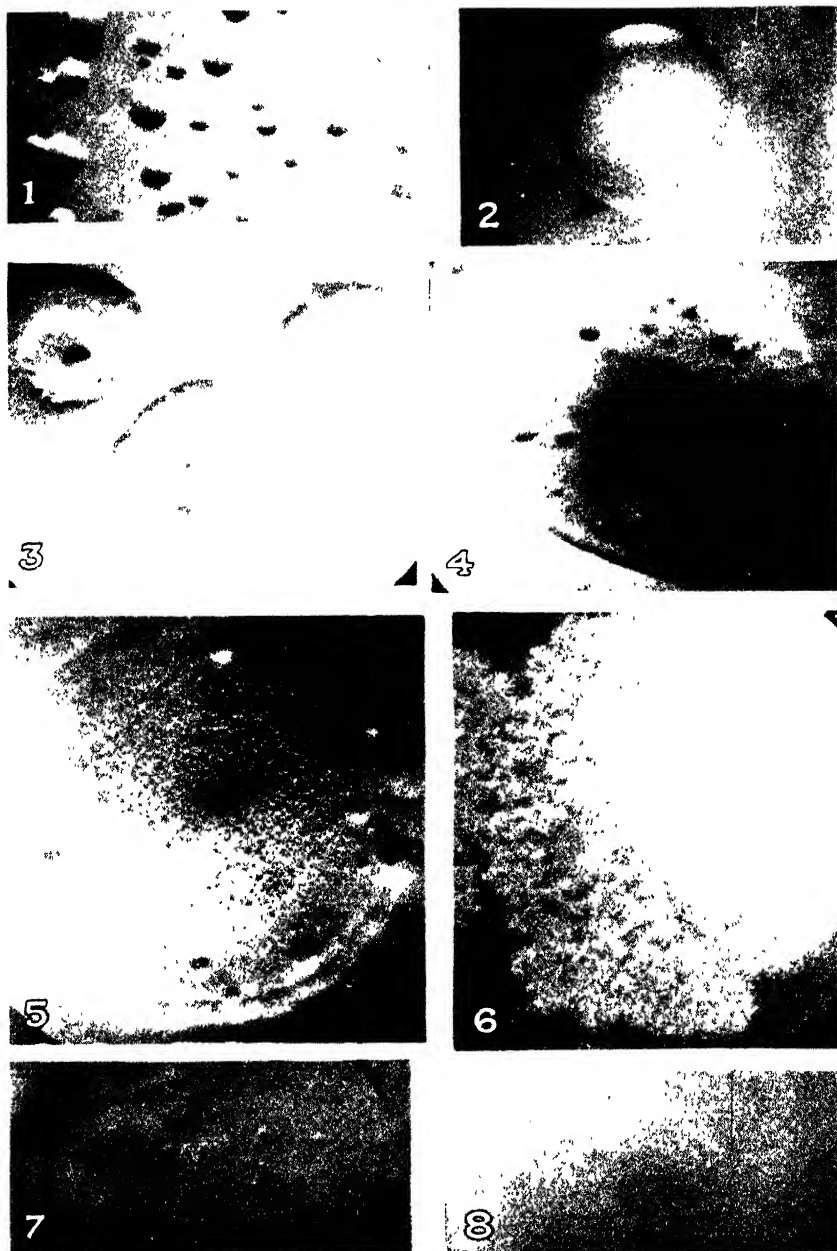
As a basis for future work some of the more common colony variations in streptococcus colonies have been described. Little concerning the sequence of the colony types is clear, but it is apparent that most of the colony variations that have been described for other bacterial species occur also in this group. The S type colony is clear cut, while the R type is not so definite. There are many intermediate types. In fluid media agglutination is associated with the R types. Certain variations also parallel the colony types, diplococcic, Gram-positive, regular cells being of the S type; while long chained, irregular, Gram-variable forms are of the R type. In natural infections, virulence



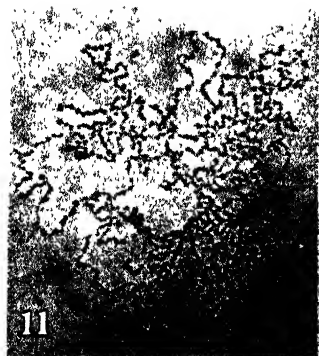
seems also to parallel the types, the S type being virulent, and the R types being relatively avirulent.

#### REFERENCES

- COWAN, MARY 1922 Variation phenomena in streptococci, with special reference to colony form, hemolysin production and virulence. *Brit. Jour. Exper. Path.*, **3**, 187.
- DUTTON, L. O. 1926a The probable rôle of the bacteriophage in streptococcus infections. *Jour. Lab. and Clin. Med.*, **11**, 763.
- DUTTON, L. O. 1926b Rôle of the bacteriophage in streptococcus infections. An interpretation of certain cultural characteristics. *Jour. Inf. Dis.*, **39**, 48.
- DUTTON, L. O. 1927 The occurrence of bacteriophage and variation in streptococcus colony formation. *Abstract, Jour. Bacteriol.*, **13**, 43.
- HADLEY, P. 1927 Microbic Dissociation. *Jorn. Inf. Dis.*, **40**, 1.



FIGS. 1 TO 8



FIGS. 9 TO 13

# SYNTHESIS OF VITAMIN B BY MICROÖRGANISMS

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Studies on the synthesis of vitamins have led to no general agreement as to the ability of specific microörganisms to produce vitamin B or as to the extent of this synthesis among the lower plant forms.

This lack of agreement may be attributed in part to the failure of investigators to keep clearly in mind the characteristics and properties that define a vitamin and in part to the marked differences in methods which have been employed.

There is, on the one hand, the group which has adhered rigidly to the conception of a vitamin as defined by Drummond (1924), i.e., a substance of unknown constitution neither fat, protein, carbohydrate, nor mineral salt, necessary for the life and well-being of a suitable organism. On the other hand, there is a group which has disregarded the requirements of Drummond and has used as the criterion of the presence of vitamin B, the ability of a substance to stimulate multiplication of a specific micro-organism. The yeast growth test of Williams (1919) employs the multiplication of *Saccharomyces cerevisiae*.

If we exclude from analysis results which are not in strict conformity with the definition of Drummond, we are still confronted by the use of a multiplicity of methods all purporting to detect the presence of vitamin B. The animal used may have been the rat, mouse or pigeon. In certain cases a few milligrams have constituted the entire quantity of microörganisms fed per rat per day and the ability of an organism to synthesize vitamin B has been decided upon the results obtained in such experiments. Again, the period of feeding has varied from two to several

weeks. Generally, no cognizance has been taken of the manner of feeding, or the time required by the animals to become accustomed to the taste of the microorganisms. In feeding certain microorganisms, the animals may continue to lose weight for as long as a week. In certain cases the organisms have been fed apart from the basal ration, whereas, in other cases they have been intimately mixed with the constituents of the basal ration.

Again, too little attention has been given to the freedom of the culture medium from vitamin. At the most, it should not contain more than traces of vitamin. Vitamin-free synthetic media are to be preferred.

For the purpose of this investigation Drummond's definition of a vitamin is accepted; no other conception is justifiable in the light of present knowledge and no adequate proof has been offered that vitamin B, as defined by Drummond, has any effect on the rate of multiplication of microorganisms. In fact, Werkman (1927) has shown by an analysis of experimental data on multiplication of *Azotobacter chroococcum* and *Rhizobium leguminosarum* that vitamin B does not stimulate multiplication of these organisms.

The purpose of this investigation was (1) to determine whether vitamin B is synthesized by certain bacteria, one torula and forms intermediate between molds and bacteria, (2) to choose these organisms from biologically separate groups of the lower plant forms so as to give an indication of the general occurrence of vitamin B synthesis among microorganisms, (3) to determine the variation, if any, in the ability of different strains of the same species to synthesize the B vitamin, (4) to make a quantitative study of the production of the vitamin, (5) to determine the effect of drying of the organisms on their vitamin content.

Feeding the microorganisms in testing for the vitamin has been quantitative so far as practicable. As much as 15 grams of organisms per rat per day have been fed. Care has been taken to accustom the rats to the diet and experiments have been continued until definite results were secured. The culture media have been synthetic or thoroughly extracted with 95 per cent hot alcohol to insure suitability for use. The uninoculated medium was always fed to control animals.

## LITERATURE

Among the workers using rats in determining the presence of vitamin B, were Wollman (1921) and Wollman and Vagliano (1922) who tested the Bulgarian bacillus and *Amylomucor* B for vitamins A and B. They concluded that neither vitamin A nor B was present. Slanetz (1923) using mice found that *Azotobacter chroococcum*, *Bacterium lactis-acidi*, *Bacillus mycoides*, *Serratia marcescens*, *Rhizobium leguminosarum*, *Bacillus subtilis* *Micrococcus agilis* and three other soil forms produced neither A nor B.

Cunningham (1924) fed young rats in order to test for both A and B in three strains of the tubercle bacillus. She found neither.

Pacini and Russel (1918) in some work referred to by McCollum and Simmonds (1925) as "not convincing" claim to have shown stimulation of the growth of rats by extracts of typhoid bacilli. Bierry and Portier (1918) injected under the skin and into the peritoneal cavity living cultures of some normal intestinal bacteria. Their animals showed improvement within twenty-four to forty-eight hours.

Damon (1921) has probably tested more organisms for vitamin B synthesis than any other worker who has published. His first results with para B, *Bacterium coli* and *Bacillus subtilis* were negative. In a later publication (1923) he concluded that Pfeiffer's bacillus and the timothy bacillus synthesized vitamin B but that *Bacillus adhaerens* and Friedlander's pneumobacillus were negative. Damon (1924) believed that a substance analogous to vitamin B was produced by *Mycobacterium smegmatis*, *Mycobact. phlei* and *Mycobact. moelleri*. Hunter (1923) concluded that *Azotobacter* synthesized vitamin B. Kuroya and Hosoya (1923) concluded that *Bacterium coli* was capable of synthesizing B. Hoet, Leclef and Delarue (1924) using both rats and pigeons found *Torula rosea*, and *Mycoderma cerevisiae* negative but *Monilia candida* positive.

Funk's pigeon test has been used extensively to determine the presence of vitamin B. McCollum and Simmonds (1925) gave evidence to support the view that "the pigeon test as ordinarily

carried out is without value for the specific purpose for which it has been so widely used."

Cooper (1914) fed *Bacterium coli* to pigeons on a polished rice diet but found no evidence of vitamin B. Weill, Arloing and Dufourt (1922) fed the colon bacillus and three spore formers from the intestinal tract of pigeons to pigeons on a polished rice diet. The birds died in what was considered normal time for polyneuritic pigeons.

Scheunert and Schieblich (1922, 1923) fed cultures of various bacteria to pigeons. They concluded that there was some effect due to vitamins in certain bacteria and no effect with other organisms. Eijkman, Van Hoogenhuije and Derks (1922) concluded from their experiments that *Bacterium coli* contains no antineuritic factor. They used pigeons in their work.

William's test for vitamin B depends upon the assumption that the increased multiplication of yeast cells upon the addition of alcoholic extracts of various substances is a measure of water-soluble B and that only. Souza and McCollum (1920) and Fulmer, Nelson and Sherwood (1921) have presented evidence to the effect that the yeast growth test is unreliable.

Robertson (1924) using William's yeast growth test concluded that *Bacterium coli*, *Serratia marcescens*, *Bact. proteus*, *Pseudomonas pyocyanea*, *Bacillus subtilis* and *Sarcina lutea* synthesized a vitamin.

Thjotta (1921) obtained evidence of a growth promoting substance for the influenza bacillus from *Bacillus proteus*, *Bacillus ozaenae* and Friedlander's pneumobacillus. Damon (1921), however, did not confirm these results.

#### METHODS AND MATERIAL

The authors have felt that the only adequate test for the presence of vitamin B is the effect of a substance upon the growth of young 50-gram white rats. Weighings of the rats receiving the substance to be tested, of control rats receiving the B deficient ration, and of rats receiving the basal ration made complete by the addition of 5 per cent wheat embryo or yeast, were made and growth curves over a period of several weeks compared. Metal

cages with a small quantity of shavings were used. The cages were cleaned weekly.

The basal ration consisted of alcohol extracted casein 18 parts, corn starch or dextrin, 73.3 parts, salt mixture 3.7 parts, salt-free butter fat 5 parts. The salt mixture was that of McCollum and Davis (1915) modified by the addition of 0.002 gram of KI and the substitution of ferric citrate for the lactate. The casein was continuously extracted with hot 95 per cent alcohol for seventy-two hours.

Young 45- to 50-gram rats were kept on this ration until definite symptoms of vitamin deficiency were observed before feeding of the organisms was started.

The microörganisms were generally fed moist in a separate container; at other times, they were mixed with the basal ration as indicated in the individual experiment. The organisms were generally grown on media in 18- by 40-inch pans. The media used are indicated under the experimental results with each organism. They were synthetic where practicable. Where peptone or agar was used, each had been continuously extracted with hot 95 per cent alcohol for never less than one week. The media were always fed to control animals as a check.

#### EXPERIMENTAL RESULTS

##### *Torula rosea*

*Torula rosea* from the stock culture collection of the department was grown on medium E of Nelson, Fulmer and Cessna (1921), modified by the use of alcohol extracted agar and sucrose. Surface growth from the pans was scraped off after three days and fed moist. In the feeding of *Torula rosea* mixed with the basal ration, it was found that the rats consistently refused to eat appreciably of the mixture.

When the torula was fed separately, the rats ate it readily. This may account for the fact that *Torula rosea* is reported in the literature as not synthesizing vitamin B. By actual determination it was found when the torula was mixed in the basal ration,



the rats received approximately 2 grams per rat per week, an amount too small to supply the vitamin need of the animal.

In figure 2 the animals received the mixed ration from the third until the end of the fifth week. Beginning with the sixth

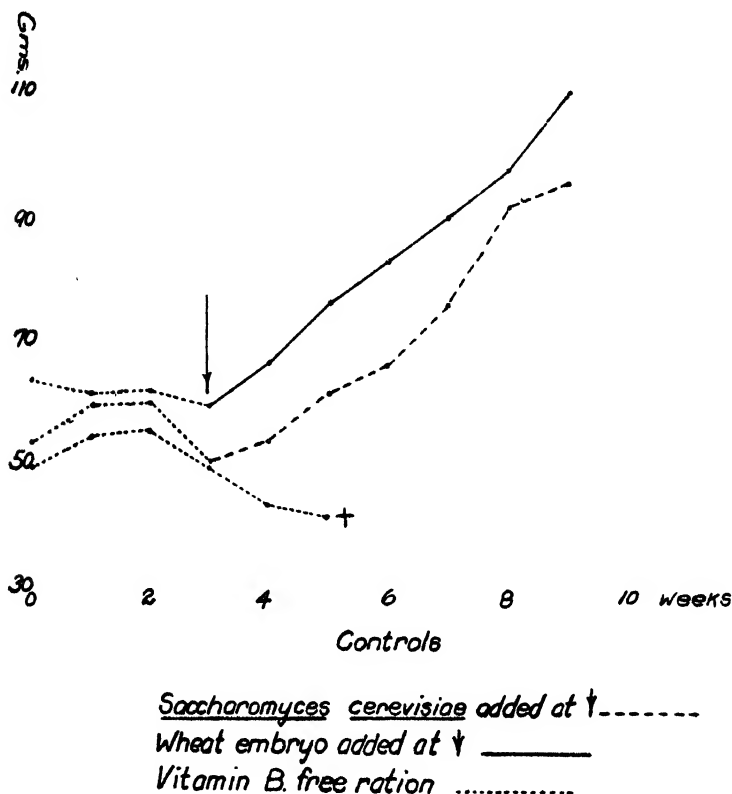


FIG. 1. EFFECT OF *Saccharomyces cerevisiae* UPON THE GROWTH OF VITAMIN B DEFICIENT RATS

Organism feeding started at ↓

week the torula was fed separately at the rate of 8 grams per rat per day. One animal was in a moribund condition and died. The remaining two began to gain in weight immediately. A gain of approximately a gram per rat per day body weight is recorded for each day torula was fed apart from the basal ration.

*Oöspora lactis*

No record of the synthesis of vitamin B by *Oöspora lactis* has been found. We have found it a rich source of the vitamin.

The organism was grown on a synthetic medium:

	grams
K <sub>2</sub> HPO <sub>4</sub> .....	2.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> .....	2.0
Succinic acid.....	0.5
CaCl <sub>2</sub> .....	0.1
FeCl <sub>3</sub> .....	0.1
MgSO <sub>4</sub> .....	0.2
Glucose (extracted).....	15.0
Distilled water.....	1000.0



FIG. 2. EFFECT OF *Torula rosea* UPON THE GROWTH OF VITAMIN B DEFICIENT RATS

Organism feeding started at ↓

This medium gave a heavy pellicle growth within four days, which could be removed *en masse*. Quantitative feeding experiments were made with this organism to determine the requirements of rats. One of several experiments is given in figure 3. The organism was fed at the rate of 2.5 grams per rat per day during the sixth week. It was then fed *ad libitum* during the seventh week when the rats consumed approximately fifteen grams per rat per day. During the eighth week it was again fed in amounts of 2.5 grams and *ad libitum* during the ninth and

tenth weeks. When fed at the rate of 2.5 grams per rat per day the animals made no gain and if larger quantities had not been tried *Oöspora lactis* would have been considered incapable of B synthesis. Larger quantities fed to the same animals gave excellent gains. *Oöspora lactis* is a good source of vitamin B, distinctly better than *Torula rosea*.

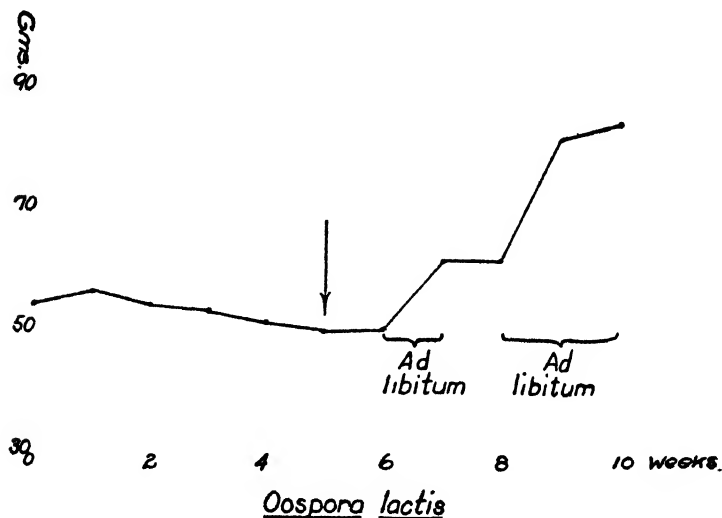


FIG. 3. EFFECT OF *Oöspora lactis* UPON THE GROWTH OF VITAMIN B DEFICIENT RATS

Organism feeding started at ↓

### *Bacillus adhaerens*

*Bacillus adhaerens* was chosen primarily because we were desirous of employing in certain experimental work an organism incapable of synthesizing vitamin B and this one had been so reported in the literature.

The organism was grown on extracted peptone agar in large petri dishes. The composition of the medium was:

	grams
Alcohol extracted peptone.....	7.0
Alcohol extracted agar.....	15.0
K <sub>2</sub> HPO <sub>4</sub> .....	2.0
Distilled water.....	1000.0

The growth was scraped from the medium after forty-eight hours incubation and fed separately. Three grams per rat per day were given to the rats in the experiment shown in figure 5. In this particular experiment after feeding the organisms for two weeks they were omitted from the ration. A drop from 16 to 2.6 grams occurred in the average gain per rat per week. *Bacillus adhaerens* is an excellent synthesizer of vitamin B.

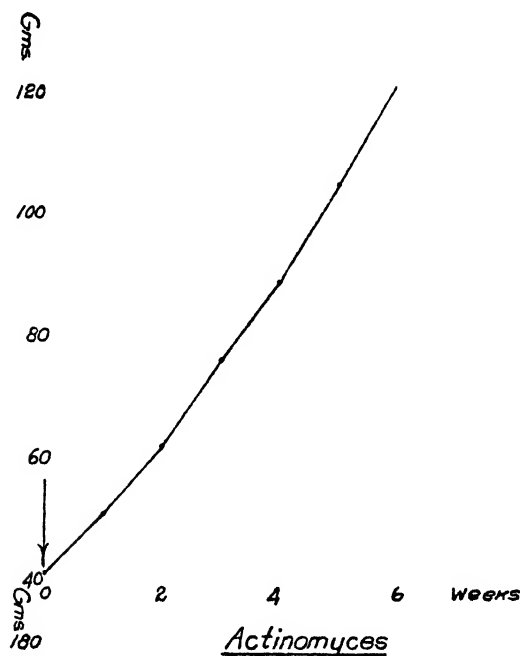


FIG. 4. EFFECT OF *Actinomyces* UPON THE GROWTH OF VITAMIN B DEFICIENT RATS  
Organism feeding started at ↓

### *Bacterium coli*

Three strains of *Bacterium coli* were tested in this experiment to determine the relative potencies of the different strains. Four grams per rat per day were fed separately. The results demonstrated that *Bacterium coli* is capable of synthesizing vitamin B and that no significant differences exist in the ability of the different strains to perform the synthesis. A growth

curve showing the average increase in weight of three rats fed strain no. 24 is shown in figure 5.

*Bacillus subtilis*

The organism was grown on extracted peptone agar, scraped off and fed apart from the basal ration. In the experiment graphed in figure 5 the rats received 6 grams per rat per day until the eighth week when they received 1 gram per rat per day.

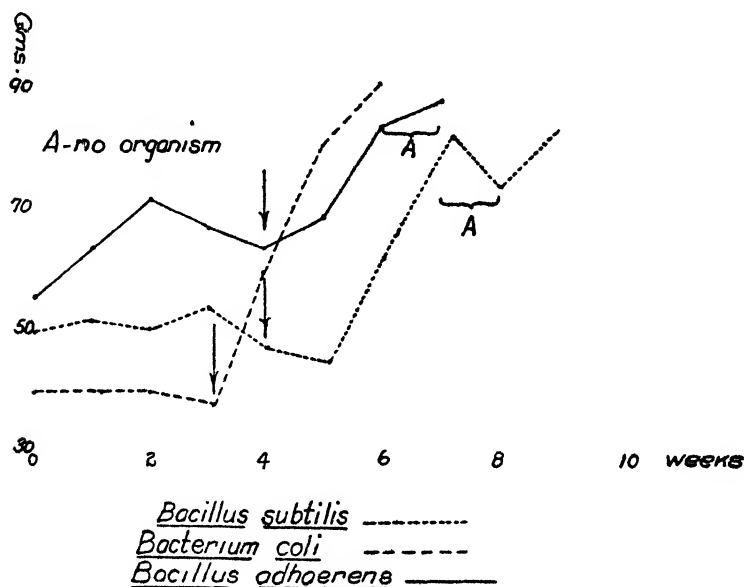


FIG. 5. EFFECT OF BACTERIA UPON THE GROWTH OF VITAMIN B DEFICIENT RATS  
Organism feeding started at ↓

In order to demonstrate that the marked loss of weight resulting was due to insufficient vitamin, the rats were again fed 6 grams per rat per day during the ninth week. Although *Bacillus subtilis* serves as an excellent source of vitamin B, one gram per rat per day of the microorganism is insufficient to maintain body weight.

Drying the bacterial growth at 37°C. for two days had no detectable effect on the vitamin potency.

*Bacillus mycoides*

Quantitative feeding results with *Bacillus mycoides* are given to demonstrate the effects of feeding various amounts of an

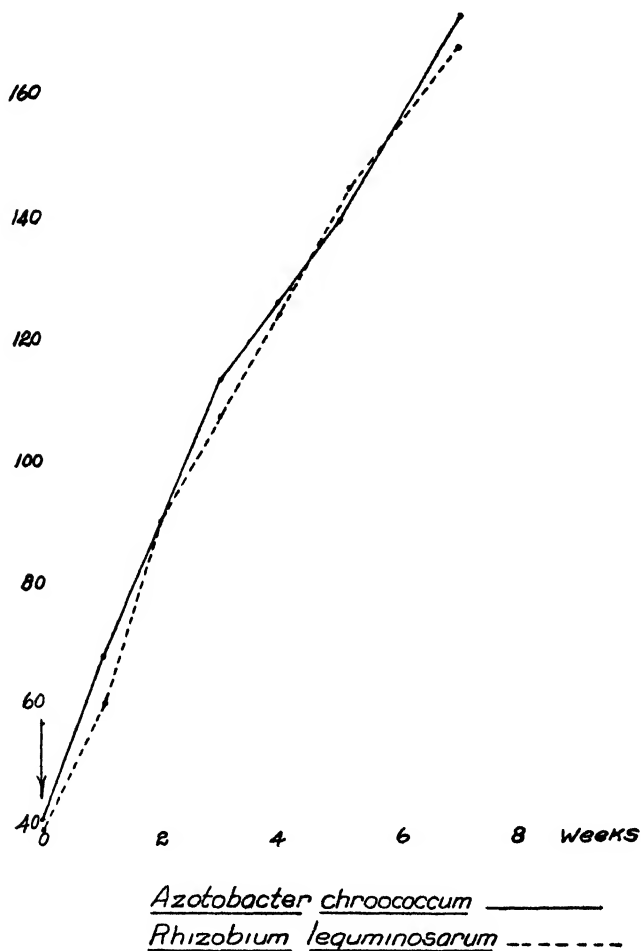


FIG. 6. EFFECT OF BACTERIA UPON THE GROWTH OF VITAMIN B DEFICIENT RATS  
 Organism feeding started at ↓

organism. It is apparent that if 2 grams or less per rat per day of mycoides were fed, the conclusion might be drawn that the organism is unable to synthesize vitamin B, whereas, our results

demonstrate that *mycoides* is a good source of vitamin B when compared with other organisms. In the results of the experiment given in figure 7, groups of rats were fed simultaneously 0.5, 1.0, 2.0, 4.0 and 8.0 grams of *B. mycoides* per rat per day. The

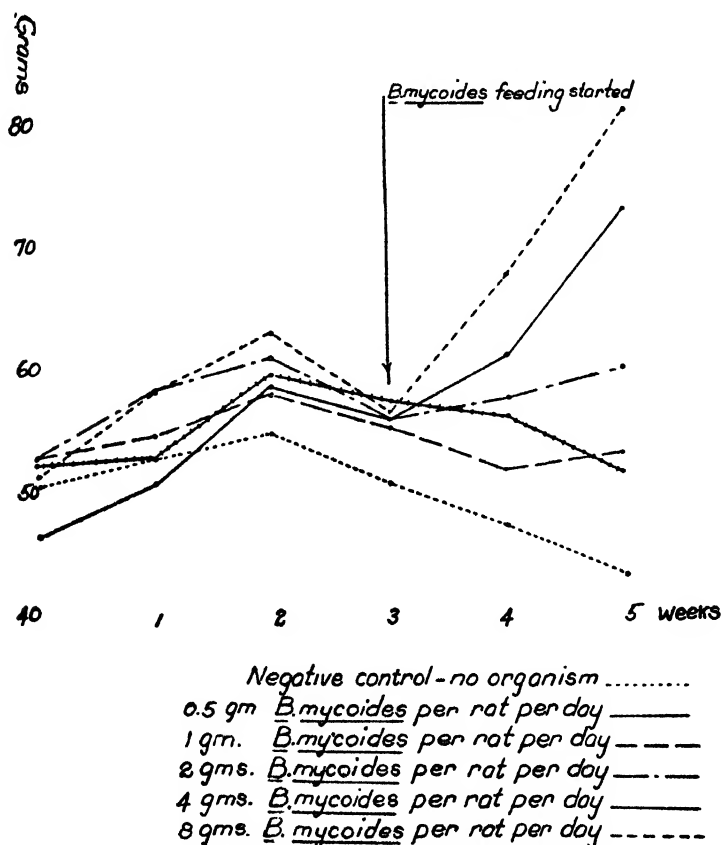


FIG. 7. GROWTH OF THE WHITE RAT ON VITAMIN B DEFICIENT RATION AND VARYING AMOUNTS OF *BACILLUS MYCOIDES*

group receiving 0.5 gram per rat per day lost weight just slightly less rapidly than the rats receiving no organisms; while the group receiving 1.0 gram per rat per day in two week's time averaged a slight loss of weight. The groups receiving 2.0, 4.0 and 8.0 grams gained in order of increasing quantity fed.

During the third week of the feeding of the organism (fig. 8) the rats that had been receiving 8.0 grams were given 2.0 grams and vice versa. The rats that had received 2.0 grams per rat per day had made an average gain of 2 grams per rat per week.

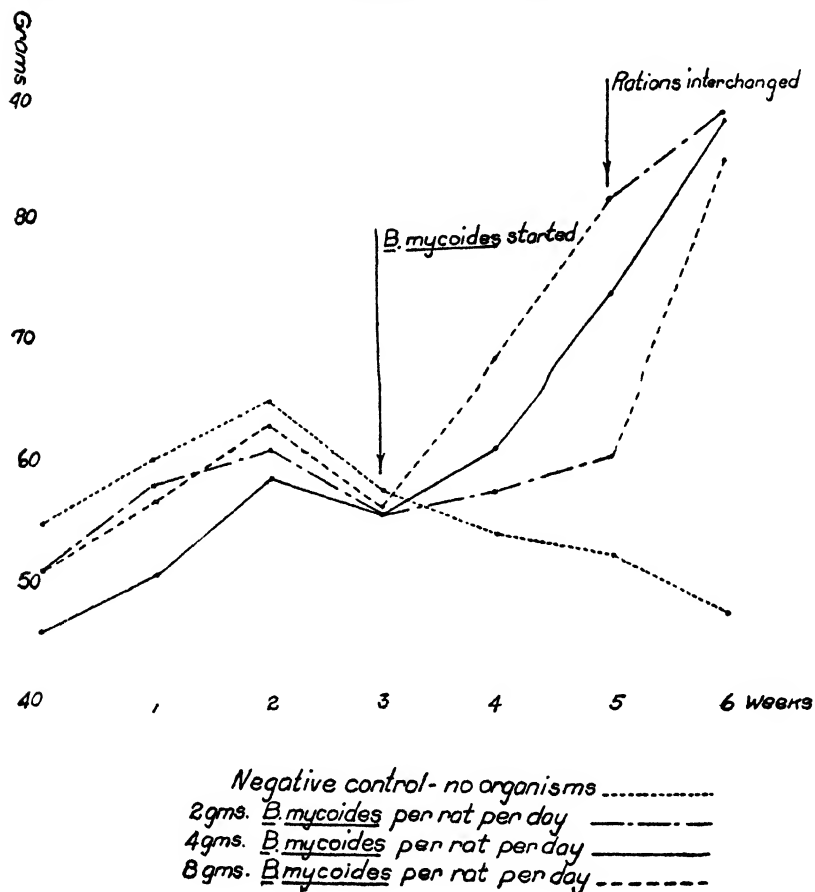


FIG. 8. GROWTH OF THE WHITE RAT ON VITAMIN B DEFICIENT RATION AND 2 GRAMS *B. MYCOIDES* INTERCHANGED WITH 8 GRAMS

When fed 8.0 grams per rat per day they made an average gain of 24 grams in one week. This marked increase can be accounted for only as due to the increase in the amount of the organism fed.

The rats that had been given 8.0 grams per rat per day made



an average gain for the two weeks of 12 grams per week. When reduced to 2.0 grams the average gain per week was 7 grams.

Organisms dried at 37°C. and at 100°C. for forty-eight hours were fed in order to determine the effect of desiccation on the vitamin potency. The results indicated no effect of drying at 37°C. and only a slight effect at 100°C.

*Azotobacter chroococcum*

This organism was grown on Ashby's medium modified by the use of alcohol extracted sucrose. The animals ate readily of the bacterial growth when mixed with the basal ration or when fed separately. In figure 6 are shown the results of feeding 6 grams per rat per day separately. Two grams per rat per day were found to be inadequate to provide sufficient vitamin B for increase in body weight.

*Rhizobium leguminosarum*

*Rhizobium* was grown on Ashby's medium modified as for *Azotobacter*. The strain happened to be one isolated from alfalfa roots. Synthesis of vitamin B by this organism is indicated by the growth curve in figure 6.

*Actinomyces (species unknown)*

An unknown soil actinomyces was grown on the following medium:

	<i>grams</i>
K <sub>2</sub> HPO <sub>4</sub> .....	1.0
KNO <sub>3</sub> .....	2.0
Calcium succinate.....	10.0
Extracted agar.....	17.0
NH <sub>4</sub> Cl.....	1.8
Distilled water.....	1000.0

Considerable difficulty was experienced in obtaining sufficient growth to feed. Long periods of incubation were necessary so that the growths scraped from large plate cultures were stored for several days before the experiment began in order to have sufficient growth to complete the feeding. Six grams per rat per day

to 2 rats constituted the only work done with actinomyces. The growth curve is shown in figure 4. The results indicate a vitamin B synthesis by the organism.

#### SUMMARY AND CONCLUSIONS

The synthesis of vitamin B by such biologically separated genera of microörganisms as *Torula*, *Oöspora*, *Actinomyces* and four genera of the order *Eubacteriales* reveals a general occurrence of vitamin B synthesis among widely separated groups of the lower plant forms. Vitamin B, whatever its structure chemically may be, is a constituent prevalent in microörganisms.

Specifically the following organisms were found to produce the growth promoting vitamin for white rats: *Torula rosea*, *Oospora lactis*, *Bacillus adhaerens*, *Bacterium coli*, *Bacillus subtilis*, *Bacillus mycoides*, *Azotobacter chroococcum*, *Rhizobium leguminosarum* and *Actinomyces* (species unknown).

The results showed no marked differences in the ability of three strains of *Bacterium coli* to synthesize vitamin B.

Drying at 37°C. or at 100°C. for forty-eight hours does not materially diminish the vitamin potency of the bacterial mass.

Several reasons are suggested to account for the discrepancies present in the literature dealing with the synthesis of vitamin B by microörganisms. Our conceptions of a vitamin are not in accord and thus differently defined the term vitamin assumes an ambiguous and obscured meaning until it is in direct conflict with the original conception intended.

After limiting by definition the meaning of the term vitamin to the legitimate conception expressed by Drummond we find a multiplicity of methods employed to determine the synthesis of a vitamin by microörganisms. Important among these are (1) quantity of organism fed, (2) period of feeding and time allowed for the rats to become accustomed to the bacterial diet, (3) manner of feeding organisms to the rats, (4) species of animal used as an indicator.

## REFERENCES

- BIERRY, HENRI, ET PORTIER, PAUL 1918 Vitamines et symbiotes. Compt. Rend. Hebdom. d. seanc. de l'acad. d. scienc., **166**, 963.
- COOPER, E. A. 1914 On the protective and curative properties of certain food stuffs against polyneuritis induced in birds by a diet of polished rice. Jour. Hyg., **14**, 12.
- CUNNINGHAM, RUBY L. 1924 An attempt to determine the presence of vitamin A and B in tubercle bacilli. Amer. Rev. Tuberc., **9**, 487.
- DAMON, SAMUEL R. 1921 Bacteria as a source of water soluble B vitamin. Jour. Biol. Chem., **48**, 379.
- DAMON, SAMUEL R. 1923 Observations on the growth of the influenza bacillus. Amer. Jour. Hyg., **3**, 247.
- DAMON, SAMUEL R. 1923 Some observations in regard to growth-promoting substances of bacterial origin. Jour. Biol. Chem., **56**, 895.
- DAMON, SAMUEL R. 1924 Acid-fast bacteria as a source of vitamin B. Jour. Path. and Bact., **27**, 163.
- DRUMMOND, J. C. 1924 Some modern tendencies of vitamin research. Chem. and Ind., **43**, 908.
- EJNKMAN, C. C., VAN HOOGENHUIJZE, J. C., AND DERKS, T. J. G. 1922 The vitamin content of microorganisms in relation to the composition of culture medium. The vitamin content of yeast. Jour. Biol. Chem., **50**, 311.
- FULMER, ELLIS I., NELSON, VICTOR E., AND SHERWOOD, F. F. 1921 The nutritional requirements of yeast. I. The rôle of vitamins in the growth of yeast. Jour. Amer. Chem. Soc., **43**, 186.
- HOET, J., LECLEF, G., AND DELARUE, G. 1924 Recherches sur la synthese des vitamines B par les microorganismes. Arch. intern. physiol., **23**, 284.
- HUNTER, O. W. 1923 Production of a growth-promoting substance by *Azotobacter*. Jour. Agr. Research, **23**, 825.
- KUROYA, DR. MASAHIKO, AND HISOYA, DR. SEIGO 1923 The synthesis of water-soluble vitamin by coli bacillus grown on synthetic medium. Scientific reports from the Government Institute for Infectious Diseases, Tokyo Imperial Univ., **2**, 287.
- MCCOLLUM, E. V., AND DAVIS, MARGUERITE 1915 Nutrition with purified food substances. Jour. Biol. Chem., **20**, 641.
- MCCOLLUM AND SIMMONDS 1925 The Newer Knowledge of Nutrition. The Macmillan Company.
- NELSON, V. E., FULMER, ELLIS I., AND CESSNA, RUTH 1921 The nutritional requirements of yeast. III. The synthesis of water-soluble B by yeast. Jour. Biol. Chem., **46**, 77.
- PACINI, AUGUST J. P., AND RUSSEL, DOROTHY WRIGHT 1918 The presence of a growth-producing substance in cultures of typhoid bacilli. Jour. Biol. Chem., **34**, 43.
- ROBERTSON, R. C. 1924 Food accessory factors (vitamins) in bacterial growth. VIII. Relations of substances formed by *B. coli* to the growth of yeast. Jour. Infect. Dis., **34**, 395.

- ROBERTSON, R. C. 1924 Food accessory factors (vitamins) in bacterial growth. IX. Growth of several common bacteria in a synthetic medium and relation of substances formed by them to the growth of yeast. Jour. Infect. Dis., **35**, 311.
- SCHEUNERT, ARTHUR, AND SCHIEBLICH, MARTIN 1922 Studien über die Magendarmflora polyneuritischer Tauben und die Bildung antineuritischen Vitamins durch Darmbakterien. Centralblatt. Bakt., I, Orig., **88**, 290.
- SCHEUNERT, ARTHUR, AND SCHIEBLICH, MARTIN 1923 Zur Kenntnis der Vitamine, II Mitteilung. Über die Bildung von Vitamine B durch obligate Darmbakterien. Biochem. Zeitschrift., **57**, 139.
- SLANETZ, E. J. 1923 Bacteria as a source of vitamins. Proceedings of the Connecticut Branch. Soc. Amer. Bact. Abs. Bact., **7**, 352.
- SOUZA, GERALDO DE PAULA AND MCCOLLUM, E. V. 1920 A study of the factors which interfere with the use of yeast as a test organism for the antineuritic substances. Jour. Biol. Chem., **44**, 113.
- THJOTTA, THEODOR 1921 Studies on bacterial nutrition. I. Growth of *Bacillus influenzae* in hemaglobin-free media. Jour. Exper. Med., **33**, 763.
- WEILL, E., ARLOING, F., ET DUFOURT, A. 1922 Essai de traitement de la carence du pigeon par des cultures mortes ou vivantes de microbes intestinaux. Compt. rend. Soc. de Biol., **87**, 50.
- WERKMAN, C. H. 1927 Vitamin effects in the physiology of microorganisms. J. Bact., **14**, 335.
- WILLIAMS, ROGER J. 1919 The vitamin requirement of yeast. A simple biological test for vitamin. Jour. Biol. Chem., **38**, 465.
- WOLLMAN, E. 1921 Sur le rôle des microorganismes dans la production des vitamines. Compt. rend. Soc. de Biol., **85**, 801.
- WOLLMAN, E., ET VAGLIANA, M. 1922 Sur le rôle des microorganismes dans la production des vitamines. Recherches sur la production des vitamines de croissance par la *Bacille bulgare* et l'*amylomucor* B. Compt. rend. Soc. de Biol., **86**, 832.



# DESCRIPTION OF AN ORGANISM FOUND IN SOLUTIONS OF SODIUM CASEINATE

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In recent measurements of the electrical conductivity of solutions of sodium caseinate (Gahl and Greves, 1926; Gahl, Greenberg and Schmidt, 1926) it was noted that the time element entered to some extent. While sufficient time had to be allowed for the establishment of heat and chemical equilibrium of the fresh solutions, it was found that this can be over-done, because such solutions undergo a gradual change in conductivity which Robertson (1910) who observed it attributed to hydrolysis of the protein.

When the cells containing the casein solution had been heated in a steam sterilizer, the resulting casein solutions did not change in conductivity when standing for several days. As a matter of fact much lower heating was sufficient to accomplish the same purpose. When a cell that was rapidly losing resistance was heated, it stopped losing and kept the resistance at practically the same point for the period following.

The behavior of the solutions in the cases just discussed can hardly be explained by anything but action of enzymes or bacteria, and actually bacteria could be plated out in large numbers in all cases in which the solutions had not been heated, but in no case were living bacteria found in the solutions which had been heated with the one exception, namely, where the heating had only been carried to 60°C. In that case a few spore-forming bacteria were found. The bacteria that were so plentiful in the unheated solutions were Gram-negative rods of uniform size and appearance, and apparently (with the exception just noted) represented a pure culture.

The question arises, whether the bacillus found in the solutions is responsible for the change in conductivity. That the change is due to hydrolysis is hardly doubtful. If this is caused by chemical factors, there is no reason for questioning Robertson's explanation. If it is caused by the bacillus, the same explanation holds true, as the bacillus produces hydrolysis.

That the hydrolysis is largely due to bacterial action is made probable by the fact that a sodium caseinate solution, the conductivity of which has been made stationary by heat treatment, begins to gain in conductivity again on injection of the bacillus into the solution. There is, however, no adequate reason to doubt that heating produces hydrolysis of a sodium caseinate solution by itself unassisted by bacteria or enzymes. In fact, the observations made in the tests referred to, that a solution always gains in conductivity by heating and cooling again, support this assumption.

The characteristics of the bacillus that I isolated are listed on the attached table in the manner recommended by the Committee on Bacteriologic Technic of the Society of American Bacteriologists (1924). I do not know if the organism occurs more or less universally. While it was traced back to the supply of the doubly distilled water that was used for the measurements and was also encountered in ordinary distilled water and tap water, it is doubtful if it can be isolated as easily from other water supplies. A few water samples from various parts of California that were sent to the State Hygienic Laboratory were examined for organisms that decompose sodium caseinate solutions and were found, it is true, to contain organisms which multiply in such solution; but they fermented carbohydrates, while the organism encountered here is entirely indifferent to carbohydrates and, judging from the alkaline reaction that it imparts to any carbohydrate broth media and to milk, attacks the proteins alone when mixed with carbohydrates.

Solutions of sodium caseinate that were left open and exposed to the air for twenty-four to forty-eight hours did not show the cloudiness that develops in a short time in solutions to which a loopful of the water in question has been added. Some slight

cloudiness resulted after a week or two, but in no case could the organism that was encountered in the water be found in these solutions that had had free contact with the air. This seems to prove that it is not disseminated by the air.

On looking up the literature in order to establish if this organism had been encountered and described before, I found that Castellani and Chalmers (1919), in their book on tropical medicine published in 1919, list a Gram-negative rod in their proteus group which is indifferent to carbohydrates. The only other characteristics given are positive motility, liquefaction of gelatine and of coagulated serum and the imparting of an alkaline reaction to litmus milk. The bacillus, named *Proteus meta-diffuens* is also listed in Castellani and Chalmer's compilation of the aerobic bacilli of the human intestine (1920) in the Annales de l'Institut Pasteur of 1920.

The reactions quoted agree with those of the organism under consideration except that the latter not only makes litmus milk alkaline but also peptonizes it and reduces the litmus.

Bergey (1923) has two genera into which the bacillus might fit, namely, the genus *Achromobacter* of the tribe Acromobacterae and the genus *Alcaligenes* of the tribe Bacterae.

*Achromobacter nebulosum* and also *A. geniculatum* closely resemble the organism under consideration inasmuch as they are Gram-negative rods with polar flagella, make milk alkaline and reduce litmus, but they do not reduce nitrates and are medium sized instead of small. The description of their characteristics does not mention whether or not milk is peptonized nor their relation to carbohydrates.

On the other hand, *Alcaligenes bookeri* and *Alcaligenes recti* also have very nearly the same characteristics as our organism. They are Gram-negative rods, 1.5 to 2.0 microns long, 0.5 microns in diameter. They are motile (by means of peritrichous flagella, however), indifferent to carbohydrates, liquefy gelatin and make litmus milk alkaline, *A. bookeri* with and *A. recti* without peptonization. *Alcaligenes recti*, therefore, corresponds to Castellani and Chalmer's *P. metadiffuens* (with the possible exception of liquefaction of serum which is not reported for *Alcaligenes recti*)



## CHARACTERISATION OF ORGANISM

*Classification according to optimum conditions: Series I (good growth in twenty-four hours at 37°C. in glucose broth)*

Index number: 5020-U1120-0000

BRIEF CHARACTERISATION	REMARKS
<p>Microscopic features:</p> <p>Form: 5 (rods)</p> <p>Endospores: 0 (absent)</p> <p>Flagella: 2 (polar)</p> <p>Gram stain: 0 (negative)</p> <p>Miscellaneous biochemical reactions:</p> <p>Biologic relationship: U (undetermined)</p> <p>Relation to oxygen: 1 (strict aerobe)</p> <p>Gelatin liquefaction: 1 (positive)</p> <p>In nitrate media: 2 (nitrite but no gas)</p> <p>Chromogenesia: 0 (none except as noted opposite)</p> <p>Carbohydrate reactions:</p> <p>Dextratic action: 0 (negative)</p> <p>From d-glucose: 0 (no acid)</p> <p>From lactose: 0 (no acid)</p> <p>From sucrose: 0 (no acid)</p>	<p>Dimensions under "Vegetative Cells."</p> <p>Heating a culture a week old to 85°C. for 10 minutes kills the organism.</p> <p>Cultures both in broth and on agar are motile. Staining according to Plimmer and Paine shows one flagellum, in polar position.</p> <p>Staining by gentian violet and counterstaining by carbol-fuchsin classifies the organism as gram negative. It is gram negative after one day's incubation and remains so.</p> <p>As the organism is strictly aerobic, it cannot be pathogenic comp. Zinseer and Russell, textbook of bacteriology, 1924, p. 28 and p. 232.</p> <p>Anaerobic culture was attempted by Burri tube, Wright-Hall and Noguichi methods. The result was negative in each case. Media used: agar and liver infusion agar, temperature 37°C. Presence of glucose or nitrate did not permit anaerobic growth.</p> <p>The aerobic character of the organism is also indicated by ordinary broth cultures, as growth in the absence of agitation is confined to the neighborhood of the surface.</p> <p>Time required for liquefaction under "Gelatin Colonies" (Growth and Gelatin Stab).</p> <p>Nitrite is decidedly in evidence after a few hours incubation at 37°C. and is also formed at room temperature. Older cultures (cultures up to 10 days were tested) also show nitrite.</p> <p>A strong brown color was observed after 4 days incubation in nitrate broth at 37° in the top layer containing the growth. At room temperature only a weak yellow-brown color resulted after 7 days incubation. Old agar colonies are yellowish and gelatin colonies brownish.</p> <p>Neither acid nor gas formation in any of the carbohydrate media tested. Decided formation of alkali in all of them, see below</p>

Observed change of pH of carbohydrate media

	glucose	asccharose	lactose	maltose	laevulose	mannite	dextrin
At inoculation	6.86	7.5	7.4	7.0	6.8	7.1	7.3
After 1 day	7.18						
After 2 days	7.71						
After 4 days	8.15	8.3	8.4	7.6	7.6	8.3	8.4
After 8 days	8.36						
After 16 days	8.80						

The fact that alkali is produced with each of these media suggests the conclusion that the organism in question decomposes the protein in the medium alone without attacking the sugars.

Rods of an average length of 1.1 $\mu$  and a diameter of 0.3 $\mu$ . Max. length 1.3 $\mu$ . End slightly rounded or tapering. Agar cultures incubated 18 hours at 37°C. were used for examination.

Chains of two or three occur, chains of four may occasionally occur.

No capsules could be proven by Hiss' staining method.

Compare "Microscopic features, endospores," for proof of non-existence of spores.

Other characteristics of agar stroke: opaque, no chromogenesis, no odor, butyrous consistency, medium unchanged

Other characteristics of agar colonies: growth rapid, colonies smooth, convex, edge entire, amorphous, young colonies white, old colonies yellowish.

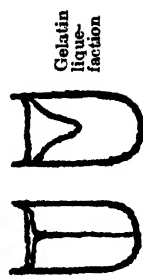
Colonies larger than punctiform are not observed, because liquefaction sets in, before they grow larger.

Growth slow at ice-box temperature (rapid at higher temperatures), colonies smooth, convex, edge entire, liquefaction saucer shaped, internal structure amorphous, color slightly brownish but translucent.

Profile of a gelatin colony



Growth on gelatin slab best at top, line of puncture filiform, liquefaction first crateriform, later infundibuliform (see sketches)  
10% liquefaction was observed after 40 hours incubation at room temperature, complete liquefaction after 48 hours



#### **Vegetative cells:**

Diameter: 1 (under 0.5 $\mu$ )

Length: 2 (more than 2 diameters)

Chains (4 or more cells)- X (doubtful)

Capsules: 0 (absent)

#### **Spores:**

Shape: — (spores do not occur)

Diameter: — (spores do not occur)

#### **Cultural features:**

Agar stroke

Growth: 1 (abundant)

Lustre: 1 (dilatant)

Surface: 1 (smooth)

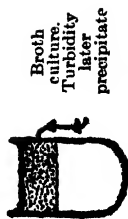
Agar colonies: 1-2 (punctiform-circular)

Gelatin colonies: 1 (punctiform)

#### **Gelatin stab:**

## CHARACTERISATION OF ORGANISM—continued

BRIEF CHARACTERISATION	REMARKS
Nutrient broth:	Growth in undisturbed medium takes place in a zone near the surface only, strong clouding, no odor, later abundant flocculent precipitate, see sketch
Temperature relations of growth:	In broth (Dunham or plain) the organism grows somewhat better at 37° than at 28°. It grows even at ice-box temperatures, say of 10°, however.
Relation of growth to reaction of medium:	The organism grew on Dunham broth between pH = 5 and pH = 11. The optimum for growth seemed to be rather below than above pH = 7, however.
Milk:	Peptonization was noticeable after 4 days incubation at 37°. Litmus, when added to milk, began to be reduced in one day, reduction was complete in two days. Methylene blue was reduced in from 5-10 hours.
Acid: 0 (no acid)	Reaction of milk: At inoculation: 6 34
Rennet curd: 0 (absent)	After 1 day incubation at 37°C. 6 49
Peptonization: 1 (present)	After 2 days incubation at 37°C. 6 56
	After 4 days incubation at 37°C. 6 86
	After 7 days incubation at 37°C. 7 40
	After 10 days incubation at 37°C. 7 86
Additional reactions:	No reaction by Salkowski, Ehrlich and vanillin tests on culture in Dunham broth, 4 days old. Blackening of lead acetate agar was noticeable after two days incubation at 37°C. Vapor from a heated broth culture turns litmus paper blue.
Indol production: 0 (negative)	It looked, as if there were a light colored colony around the top of the needle hole.
Production of hydrogen sulfide: positive	Greenspoon's medium shows beginning liquefaction after two days incubation at 37°C.
Production of ammonia: positive	The organism was found alive in Uchinsky medium a month after inoculation. It seemed, as if it had grown to some extent, but this was not established with certainty.
Methyl red test: negative	Gelatin liquefied by organism, when added to gelatin containing 1% phenol, causes its liquefaction after a time depending on quantity of liquefied gelatin added.
Voges-Proskauer reaction: negative	
Potato medium: reaction doubtful	
Liquefaction of coagulated blood medium: positive	
Growth in artificial media:	
Production of proteolytic enzyme: positive	



Broth culture.  
Turbidity  
later  
precipitate

while *A. bookeri* resembles the organism described above. The most conspicuous differences are the type of flagella as mentioned before, the fact that *A. bookeri* does not reduce nitrates and that it grows luxuriantly on potato medium. I might mention here that the production of nitrites from nitrates is perhaps the most characteristic reaction of the organism that I isolated. I was able to demonstrate the presence of nitrites less than three hours after inoculation into nitrate broth, that is, earlier than growth could be observed.

I was fortunate enough to have the advice regarding the classification of this organism, of Dr. Max Levine of the Iowa State College who pointed out the close resemblance with *Pseudomonas fluorescens* (Levine and Soppeland, 1926) from which it differs, however, in its failure to produce the characteristic fluorescent pigment. This property, Doctor Levine remarks, is so variable however, that it is impossible adequately to differentiate upon it as a criterion.

While fully admitting the weight of the argument, I hesitate to classify this organism which as re-examination showed, produces nothing that can be called a pigment (except in nitrate broth; compare chart), in a group of organisms characterized by pigment production.

Dr. Aldo Castellani very kindly compared my culture with his *Proteus metadiffuens* and is under the impression that the two are identical. He also expressed his willingness to let me have a culture of *Proteus metadiffuens* so that I could study the two organisms side by side, but owing to the fact that Doctor Castellani's culture is kept abroad, it has not been possible so far to arrange this.

Although it is quite evident that the organism in question differs little from others previously described, it seems advisable to me to consider it as a separate species, as long as its identity with others is not proven. I venture, therefore, to put it into the genus *Achromobacter* and to designate it as *Achromobacter caseinicum*.

## REFERENCES

- Bergey's Manual of Descriptive Bacteriology. 1923 Baltimore, pp. 132 and 233.
- CASTELLANI, ALDO, AND CHALMERS, ALBERT J. 1919 Manual of Tropical Medicine, London, p. 950.
- CASTELLANI, ALDO, AND CHALMERS, ALBERT J. 1920 Annales de l'Institut Pasteur, **34**, 614.
- Committee on Bacteriologic Technic of the Society of American Bacteriologists, Descriptive Chart prepared by the. 1924 Presented at the annual meeting of the Society, December 29, 1924.
- GAHL, RUDOLF, AND GREVES, G. L. 1926 University of California Publications in Physiology, **5**, 289.
- GAHL, RUDOLF, GREENBERG, DAVID M., AND SCHMIDT, CARL L. A. 1926 University of California Publications in Physiology, **5**, 307.
- LEVINE, MAX, AND SOPPELAND, LULU 1926 Bacteria in creamery wastes. Iowa State College of Agriculture and Mechanic Arts. Official Publication, **24**, 36.
- ROBERTSON, T. BRAILSFORD 1910 Jour. Phys. Chem., **14**, 528.
- ROBERTSON, T. BRAILSFORD 1918 The Physical Chemistry of the Proteins. New York, London, Bombay, Calcutta and Madras, p. 456.

# THE BACTERIAL DESTRUCTION OF ACETYL-METHYL-CARBINOL

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The occurrence of strains of members of the colon-aerogenes group of bacteria which are irregular in their physiological behavior with regard to the correlation of the methyl red and Voges-Proskauer tests is well established. These irregular strains may be either positive in both tests, negative in both tests, or variable in one or both tests. That such irregular behavior is due to contaminating organisms does not appear to be probable in the light of the work of Chen and Rettger (1920) who were unable to detect contamination by repeated plating, although they did succeed in reducing the number of non-correlating strains in their series from 18 to 4 by such a procedure.

A possible explanation of the double negative reaction has been presented in the work of Paine (1927) who showed it to be very probable that certain strains of organisms of the colon-aerogenes group have the ability to destroy acetyl-methyl-carbinol. In view of the great importance which has been attached to the Voges-Proskauer reaction, both as a taxonomic character and as a criterion of habitat it appeared to us that the findings of Paine were worthy of an attempt at confirmation and of extension. We have, accordingly, checked and extended his work with a larger number of strains of members of the colon-aerogenes group, and have added representatives of other genera, although for obvious reasons our attention has been primarily focused on the former group.

## EXPERIMENTAL

The principal source of strains of colon-aerogenes bacteria was the collection of Lewis and Pittmann (1928), which was

kindly placed at our disposal by Professor Lewis. Cultures from this source were supplemented by stock cultures and by cultures freshly isolated from surface waters. A total of 113 accurately identified strains have been tested for their ability to destroy acetyl-methyl-carbinol. This series included representatives of each of the 7 species established by Levine (1921) and also a group of 3 strains which were so irregular as not to be readily classified. A total of 89 strains of green fluorescent bacteria, most of which were isolated during the summer of 1927 by Mrs. Mary K. Taylor, have also been tested as have representatives of the *Salmonella*, the *Eberthella*, the *Proteus*, the *Serratia* and the *Bacillus* genera.

The medium used for the production of acetyl-methyl-carbinol was prepared according to the formula given in the Standard Methods of the American Public Health Association, using Bacto Proteose peptone. This medium after sterilization in flasks was inoculated with culture no. 309, a typical *Aerobacter cloacae*, which had been isolated from spring water, and which gives a very strongly positive Voges-Proskauer reaction. After five days incubation at 37°C. the flasks were steamed and the dead bacteria removed either by centrifugalization or by filtration. The clarified medium was tubed in approximately 5 ml. quantities, resterilized and inoculated with the organisms to be tested. In all cases luxuriant growth was obtained in this medium. Tests for the presence of acetyl-methyl-carbinol were made at intervals up to twenty days by the usual method. Sterile controls were invariably positive. The results obtained with members of the colon-aerogenes group are summarized in table 1.

The results with other organisms may be given briefly as follows: Acetyl-methyl-carbinol was not destroyed within ten days by the following: *Salmonella schotmülleri* 4 strains; *Salmonella paratyphi*, *Salmonella suispestifer* and *Eberthella typhi*, 3 strains each; *Salmonella enteritidis*, *Salmonella aertrycke*, *Salmonella typhi-murium* and *Eberthella paradysenteriae* 2 strains each; *Salmonella morgani*, *Salmonella pestis-caviae*, *Serratia marcescens*, and *Proteus vulgaris* 1 strain each; green fluorescent bacteria 18 strains. It was destroyed by the following: *B.*

*subtilis* 5 strains; unidentified spore formers 3 strains; *B. cereus*, *B. mycoides* and *B. vulgatus* 1 strain each; green fluorescent bacteria 71 strains.

Strains of the colon-aerogenes group which were found to destroy acetyl-methyl-carbinol were selected for a more intensive study. Culture no. 151, a typical *Es. communior*, was unfortunately lost during the progress of the work and is, therefore, included only in the total number of acetyl-methyl-carbinol destroyers. Culture no. 101 is classed as an *Aero. aerogenes* on the basis of the appearance of the colony on eosin-methylene blue and Endo agars, its fermentation reactions and the fact

TABLE 1

ORGANISM	NUMBER OF STRAINS TESTED	TEST FOR ACETYL-METHYL-CARBINOL IN ACETYL-METHYL-CARBINOL POSITIVE MEDIUM	
		Number of strains	
		Positive	Negative
<i>Es. coli</i> .....	9	9	0
<i>Es. communior</i> .....	27	22	5
<i>Es. acidi-lactici</i> .....	31	31	0
<i>Es. coscoroba</i> .....	1	1	0
<i>Es. neapolitanum</i> .....	7	5	2
<i>Aero. aerogenes</i> .....	20	7	13
<i>Aero. cloacae</i> .....	15	15	0
Irregular strains .....	3	2	1

that when first isolated it gave a weakly positive Voges-Proskauer reaction. The failure to secure the same Voges-Proskauer reaction after carrying in culture for some months as when freshly isolated has been previously observed by Koser (1924).

Only one culture of this group, *Es. communior* no. 222, failed to produce the negative condition within three days, this organism requiring five days. The methyl-red reaction, the Voges-Proskauer reaction as determined in standard glucose medium at daily intervals for five days, and the rate of destruction of acetyl-methyl-carbinol in a positive medium are shown in table 2.



It is apparent that acetyl-methyl-carbinol may be produced and destroyed in the same medium by some cultures within the time limit customarily used for testing. This stands in contradiction of the statement by Levine (l. c., p. 21) that "neither the character of the medium nor the period of incubation of culture interferes seriously with the test, . . . ." Furthermore, this

TABLE 2

ORGANISM	METHYL RED REACTION	TEST FOR ACETYL-METHYL-CARBINOL IN GLUCOSE MEDIUM					TEST FOR ACETYL-METHYL-CARBINOL IN ACETYL-METHYL-CARBINOL MEDIUM						
		1 day	2 days	3 days	4 days	5 days	12 hours	18 hours	24 hours	30 hours	2 days	3 days	5 days
<i>Aero. aerogenes</i> M-6.....	-	+	±	±	-	-	+	Tr.	-	-	-	-	-
<i>Aero. aerogenes</i> M-10.....	-	-	-	-	-	±	+	±	+	+	-	-	-
<i>Aero. aerogenes</i> M-12.....	-	+	±	±	-	-	+	-	-	-	-	-	-
<i>Es. communior</i> M-16 .....	+	-	-	±	-	-	+	±	+	+	-	-	-
<i>Aero. aerogenes</i> 89.....	-	+	+	-	-	-	+	+	±	-	-	-	-
<i>Aero. aerogenes</i> 91.....	-	±	Tr.	-	-	-	+	+	±	-	-	-	-
<i>Aero. aerogenes</i> 94.....	-	+	Tr.	-	-	-	+	+	±	-	-	-	-
<i>Aero. aerogenes</i> 98.....	-	±	Tr.	-	-	-	+	+	±	-	-	-	-
<i>Aero. aerogenes</i> 100.....	-	-	Tr.	-	-	Tr.	+	+	±	-	-	-	-
<i>Aero. aerogenes</i> 101.....	-	-	-	-	-	-	+	+	±	-	-	-	-
<i>Aero. aerogenes</i> 163.....	-	+	±	-	-	-	Tr.	-	-	-	-	-	-
<i>Es. communior</i> 216.....	+	-	-	-	-	-	+	±	-	-	-	-	-
<i>Es. communior</i> 222 .....	+	-	-	-	-	-	+	+	+	+	+	-	-
<i>Aero. aerogenes</i> 294.....	-	±	Tr.	-	-	-	+	Tr.	-	-	-	-	-
<i>Aero. aerogenes</i> 311.....	-	-	-	-	-	±	+	+	+	+	-	-	-
<i>Es. communior</i> 320.....	+	-	-	-	-	-	+	+	+	+	-	-	-
<i>Es. neapolitanum</i> 328.....	+	-	-	-	-	?	Tr.	+	±	±	-	-	-
<i>Es. neapolitanum</i> 338.....	+	-	-	-	-	?	+	+	±	±	-	-	-
<i>Aero. aerogenes</i> 339.....	-	-	-	?	-	Tr.	+	+	+	+	-	-	-
Irregular 341.....	-	-	-	-	-	-	+	±	+	±	-	-	-

fact does, we believe, present a serious obstacle to the use of the Voges-Proskauer reaction as a differential test in the taxonomy of this group of bacteria.

In order to determine whether the rate of destruction of acetyl-methyl-carbinol was influenced by the addition of extra nutrient material we have tested the series of cultures in a positive medium

reinforced with glucose in amounts of 0.1, 0.3, 0.5 and 1.0 per cent, with glucose and peptone 0.5 per cent each, and with peptone alone 0.5 per cent. The results obtained may be briefly summarized. The addition of peptone alone has no effect. The destruction of acetyl-methyl-carbinol, however, is markedly influenced by the addition of glucose in amounts exceeding 0.1 per cent, in that, with the exception of one culture, it occurs only after a longer period of incubation than is required in non-reinforced controls, the delay being roughly proportional to the amount of glucose added.

In the light of this sparing action of glucose we believe that the disappearance of the acetyl-methyl-carbinol from a positive medium is due to the fact that it is utilized as a source of carbon by the organism. This opinion is supported by a determination of the reaction of the medium at intervals after inoculation with a destroying culture. The pH shifts to the alkaline range, which, in view of the work of Ayers and Rupp (1918) is interpreted as being due to the production of carbonates from the carbon compounds present. The belief of Paine that the negative reaction is not explicable on the basis of the exhaustion of the peptone necessary for the color change appears to be confirmed.

In order to determine whether strains which both produce and destroy acetyl-methyl-carbinol were "mixed," culture no. 89 was streaked from a water suspension on a number of Endo plates. Only one type of colony was observed. Random picking of 30 well isolated colonies to a Voges-Proskauer positive medium showed no variation in the ability to destroy acetyl-methyl-carbinol.

The ability to destroy acetyl-methyl-carbinol does not correlate with any other character which has been determined for the cultures used except the fermentation of sucrose, which cannot be considered as significant. Based on the utilization of citrate all except 3 of the cultures, nos. 100, 151, and 163, are non-fecal in origin. The results here reported have been consistent on repeated retesting.

## SUMMARY

Acetyl-methyl-carbinol is destroyed by certain strains of (1) the colon-aerogenes group of bacteria, chiefly *Aero. aerogenes*, (2) the green fluorescent bacteria, and (3) by all of the aerobic spore formers tested. It is not destroyed by the representatives tested of the *Salmonella*, *Eberthella*, *Proteus* or *Serratia* groups.

It seems probable that the compound serves as a source of carbon.

## REFERENCES

- American Public Health Association 1923 Standard Methods of Water Analysis.
- ATERS, S. H., AND RUPP, PHILIP 1918 Simultaneous acid and alkaline bacterial fermentation from dextrose and the salts of the organic acids, respectively. Jour. Inf. Dis., **23**, 188-216.
- CHEN, C. C., AND RETTGER, L. F. 1920 A correlation study of the colon-aerogenes group of bacteria, with special reference to the organisms occurring in the soil. Jour. Bacteriol., **5**, 253-298.
- KOSER, S. A. 1924 Correlation of citrate utilization by members of the colon-aerogenes group with other differential characteristics and with habitat. Jour. Bacteriol., **9**, 59-77.
- LEVINE, MAX 1921 Bacteria fermenting lactose and their significance in water analysis. Bull. 62, Eng. Exp. Sta., Iowa State College.
- LEWIS, I. M., AND PITTMAN, E. E. 1928 The correlation between differential tests for colon bacteria and sanitary quality of water. Jour. Amer. Water Works Assoc., **19**, 78-92.
- PAINE, F. S. 1927 The destruction of acetyl-methyl-carbinol by members of the colon-aerogenes group. Jour. Bacteriol., **13**, 269-274.





# THE ISOLATION AND ESTIMATION OF CLOSTRIDIUM WELCHII

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For some time we have been using a technic for the isolation and identification of anaerobic bacteria in feces, believing that we were able to recover all species of the genus *Clostridium*. All those isolated were identified by means of Hall's key (1922) and confirmed by the best criteria at our disposal. However, after about 100 specimens had been examined, we realized that no putrefactive anaerobes were being recovered. Investigation showed that all the anaerobes isolated were *C. Welchii* or organisms which differed from it in a few minor respects. The latter were probably atypical *C. Welchii*.

Realizing the importance of this finding, we set out to investigate the problem more thoroughly. The method for securing aerobiosis (phosphorus) was not unusual and as human blood agar was the medium used, we were at a loss to explain the phenomenon.

A résumé of the events which had led to our selection of the method may be of interest. We had set out to find a plate-count method for the study of anaerobes in feces. We thought that, if this were successfully accomplished, we could enumerate and separate the anaerobic species. We felt that the usual method of inoculating given amounts of a bacterial suspension into egg-meat or milk media had several disadvantages. In the first place, it is necessary to heat the suspension to 80°C. for fifteen minutes to kill non-sporulating bacteria. This also destroys the vegetative forms of anaerobes and in species which sporulate poorly (nearly all non-putrefactive strains) most, if not all, the anaerobes would be lost. Second, pure cultures are recovered

with difficulty. Third, as only spores give the test, this method gives but a poor idea of the total number of anaerobes present.

Our first problem was to find a method for isolating anaerobes without the preliminary heating. This would give us the total count and not merely the spores. It is well known that sporulating anaerobes will grow at temperatures up to about 55°C. and we hoped that this temperature would inhibit non-sporulating bacteria such as *B. coli*. It was found that temperatures as low

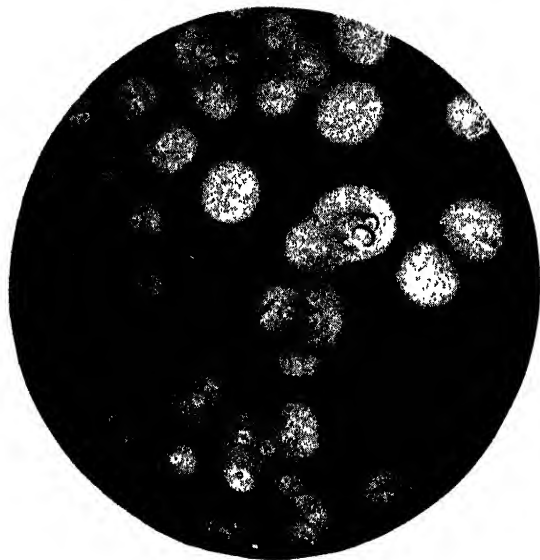


FIG. 1. FECES PLATED ON BLOOD AGAR AND GROWN AT 48° IN AN ANAEROBIC JAR

The large hemolytic colonies are *C. welchii*

as 45°C. had a marked inhibitive effect on *B. coli* but did not affect the anaerobes. In fact, many anaerobes grew better at temperatures as high as 50°C. One pleasing feature was that hemolytic types of *B. coli*, which are quite common, did not produce hemolysis in the Varney anaerobic jar (Varney, 1926).

The blood agar plate was found suitable as a "plate-count" method as most anaerobes are hemolytic. A majority of the anaerobes also produced hydrogen sulfide but the medium of Wilson and Blair (1924) which utilizes this property, gives a much lower count. All our strains of anaerobes died out in a

few days on this medium, while they were carried indefinitely on blood agar. Transplants were frequently lost on the sulfite medium. The blood agar plate has been used by Thompson (1926) for the identification of anaerobes but our results fail to confirm the differential value of the medium. In fact, the results reported by Thompson can readily be produced by varying the composition of the blood agar used. For instance, the difference observed when meat infusion is used as a base as compared with

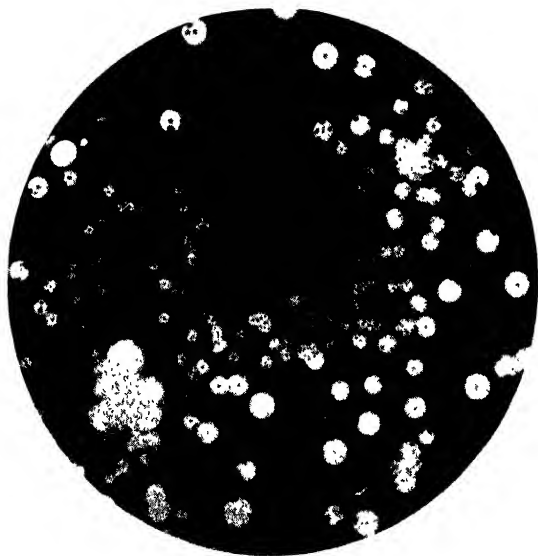


FIG. 2. THE SAME SAMPLE PLATED ON BLOOD AGAR AND GROWN AT 37° IN AIR

Note the hemolysis by *B. coli*. A number of non-hemolytic types of *B. coli* are shown.

meat extract is quite startling. There is much more hemolysis on the meat extract medium. Many of our strains of *C. Welchii* have produced dark outer zones, which is contrary to Thompson's statements. We found no uniformity in the type of hemolysis.

At this point we were impressed with Welch's method (1926) on account of its simplicity. We presumed that all anaerobes would grow in his phosphorus jar and 486 hours we proceeded to adopt it for future work, with the following modification. The phosphorus was put in a porous porcelain battery jar. This jar



is non-corrosive and allowed most of the water of condensation to escape so that the phosphorus was kept fairly dry. The battery jar was not covered. This allowed a better circulation of the spent air and consequently a more prompt exhaustion of the oxygen in the jar. There was no danger of splashing of the hot phosphorus and no accidents have occurred during one year's use of the jar.<sup>1</sup> When it is desired to open the jar to examine the contents, we have a beaker of cold water ready and pour this into the battery jar immediately after removing the lid from the

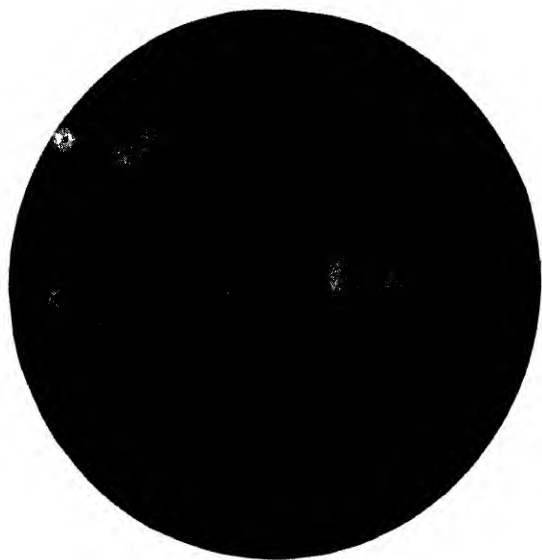


FIG. 3. BLOOD AGAR CULTURE FROM FECES SHOWING ANOTHER VARIATION IN THE HEMOLYSIS BY *C. WELCHII*

museum jar.<sup>1</sup> This removes all danger from burning phosphorus and from phosphorus pentoxide fumes which generate rapidly as soon as the phosphorus is exposed to air.

We plated 0.01 cc. of several dilutions on the surface of human blood agar plates and incubated them in this jar at 48°C. for eighteen to twenty-four hours. After about 100 examinations by this technic, we were surprised to find that the only anaerobes

<sup>1</sup> The author received second and third degree burns of both hands from neglect of this important detail.

recovered were *C. Welchii*. Investigation showed that the inhibition of the other anaerobes was largely due to the effect of the phosphorus pentoxide vapor. A number of anaerobes were

TABLE I  
*Counts of C. welchii per 1 gram dry feces*

NAME	HEATED AT 85° FOR 15 MINUTES AND GROWN AT 37°	UNHEATED GROWN AT 48°	SPORES
			<i>per cent</i>
Mrs. C. H. ....	1,200	800,000	0 15
Mrs. M. ....	70,000	390,000	18
Mrs. R. ....	30,000	140,000	21
Irwin H. ....	2,200	20,000	11
Mrs. L. ....	50,000	260,000	19
Mr. L. ....	400	4,000	10
W. S. ....	1,500,000	2,000,000	75
Miss G. S. ....	5,700	1,200,000	0 47
Mr. L. ....	600	19,000	3
Mrs. J. W. L. ....	None found	500	0
Mrs. M. H. ....	3,400	100,000	3
Mrs. W. C. ....	1,600,000	5,000,000	33
Mrs. L. V. H. ....	1,000,000	4,000,000	25
Miss E. L. ....	1,400,000	3,500,000	40
Dr. M. L. ....	2,000	20,000	11
Willard G. T. ....	1,300	3,000,000	0 04
J. J. ....	4,000,000	4,000,000	100
Mrs. W. J. S. ....	0	0	
Mrs. R. H. A. ....	0	0	
Miss F. F. ....	100	36,000	0.3
Gladys H. ....	200	20,000	1
Miss J. M. ....	21,000	1,100,000	2
F. S. A. ....	11,000	10,000	100
L. W. R. ....	0	0	
M. D. H. ....	1,100	20,000	5.5
W. L. S. ....	8,300	3,000,000	0 3
T. H. W. ....	11,000	40,000	27
Mrs. T. J. ....	1,500,000	8,000,000	0
J. G. L. ....	15,000,000	10,000,000	50
H. ....	21,000	75,000	28

secured through the courtesy of Dr. James G. Hall, and these were plated on blood agar and cultivated in the phosphorus jar at 37° and 48°C. A few produced streptococcus-like colonies at 37°C.

but only rarely did we obtain a growth at 48°C. and no hemolytic forms were observed. The exception was *C. Welchii*. There was an abundant growth and considerable hemolysis at both temperatures.

We were then anxious to learn what would happen to cultures from a specimen of feces which was heavily contaminated by facultative anaerobes, especially *B. coli*. We found that the colonies could still be recognized by their hemolysis even when the contaminating colonies covered almost the entire surface of the



FIG. 4. BLOOD AGAR CULTURE FROM FECES SHOWING *C. WELCHII*

Figures 1, 3 and 4 were grown under identical conditions

plate. The colonies are larger than the contaminating ones although the hemolysis may be reduced from the usual 1 cm. to about 2 mm. or even less. But this only happens to an exceptionally heavily contaminated plate.

To demonstrate that vegetative forms of *C. Welchii* were recovered by the above technic, several samples of feces were divided into two parts. The first was heated to 80°C. for fifteen minutes as with older methods and plated on blood agar which was then grown at 37°C. in the phosphorus jar. The second was

plated directly on blood agar without preliminary heating and grown at 48°C. in the same type of jar. Anaerobic conditions were produced as described above. Table 1 shows the results obtained.

It will be seen that the percentage of spores is extremely variable and therefore the older methods must have been unreliable for the quantitative estimation of sporulating anaerobes. We have observed quite frequently that after an exclusive diet of *L. acidophilus* milk and lactose the spores are almost, if not entirely absent, while vegetative forms of anaerobes may be present in large numbers.

As previously stated, we do not heat the suspension to 80°C. as is usually done to kill the non-sporulating organisms but rely on the temperature of incubation to prevent their growth. We prefer incubation at 48°C. because, although most of the anaerobes will grow at 50°C. or even 55°C., 48°C. is sufficiently high to eliminate to a marked extent non-sporulating forms, especially *B. coli*. This temperature will allow for the variations which commonly occur in laboratory incubators which would otherwise tend to ruin the cultures if they became too hot.

We have observed about four types of *C. Welchii* based on their appearance on the blood agar plate. The first has a clear area of hemolysis extending to the outer border of the hemolytic zone. This type is infrequently found. The second has alternating hemolytic and dark bands surrounding the colony. The third has a dark band around the single hemolytic zone. The fourth has no dark outer band.

#### SUMMARY

A method possessing the following advantages has been described for the isolation and estimation of *C. Welchii* in biological material:

- a. It is specific for *C. Welchii* as compared with other anaerobes.
- b. *C. Welchii* is readily recovered in pure culture.
- c. The numbers recovered are much larger than with other methods. The total viable bacteria are cultivated.

*d.* Preliminary heating to destroy vegetative forms is eliminated.

#### REFERENCES

- HALL, I. C. 1922 Jour. Inf. Dis , **30**, 445.  
THOMPSON, L. 1926 Jour. Bacteriol., **11**, 305.  
VARNEY, P. L. 1926 Jour. Lab. and Clin. Med., **11**, 1183.  
WILSON AND BLAIR 1924 Jour. Pathol. and Bacteriol., **37**, 119.

# STUDIES ON THE PROTEOLYTIC BACTERIA OF MILK

## I. A MEDIUM FOR THE DIRECT ISOLATION OF CASEOLYTIC MILK BACTERIA

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Various methods have been introduced for the isolation and identification of the caseolytic organisms of milk. By means of casein agar plates, which after incubation are flooded with acid, the colonies of caseolytic organisms may be noted, but the organisms cannot then be picked from the plates for further study in pure culture. The action of some organisms on the casein in these plates may be very slow and slight. Ayers (1911) recommended that plates made with his casein agar should be incubated for six days. Use has also been made of the milk agar plates of Freudenreich (1895) which are prepared by the addition of about 10 per cent of sterile milk to nutrient agar. Organisms are usually considered proteolytic if there is a clear zone about the colony. That this method is unreliable in the case of acid-forming bacteria has been pointed out by Hastings (1904), Slobodskaya-Zaykowska (1925), and others, for the clear zone about the colony may be produced by the solvent action of dilute lactic or other acid. If sufficient acid is produced there will be a zone of precipitation immediately about the colony, surrounded by a clear zone; but if little, or weak, acid is formed there may be a clear zone without any precipitate. It is therefore difficult to determine whether or not an acid-forming organism is weakly caseolytic. If the milk agar plate is flooded with dilute (1 per cent) HCl the excess of acid will precipitate the undigested casein, but it is then impossible to pick the organisms in pure culture.

In a study of proteolytic milk organisms of 230 cultures picked

as caseolytic from milk agar plates, 86 were found on further test to be non-caseolytic. Most of these produced considerable quantities of acid in milk, but 11 of them produced little acid in milk and gave a clear zone about the colony on milk agar plates. By means of the casein agar medium described below, however, 59 cultures were picked and all were found to be caseolytic in milk.

TABLE 1  
*Action of organisms on new casein-agar plates and on milk-agar plates*

ORGANISM	NUMBER OF CULTURES	NEW CASEIN-AGAR PLATE. HALO	MILK-AGAR PLATE		pH IN MILK	CASEIN DECOMPOSITION*
			Halo	Precipitate		
Control.....		—	—	—	6.6	—
<i>M. citreus</i> .....	31	+	+	—	5.6	+
<i>M. perflavus</i> .....	11	—	+	+	5.9	—
<i>M. varians</i> .....	9	+	+	+	5.4	+
<i>M. casei</i> .....	35	+	+	+ or —	5.7	+
<i>M. percitreus</i> .....	5	+	+	—	5.3	+
<i>M. luteus</i> .....	5	+	+	—	5.4	+
<i>M. cereus</i> .....	3	+	+	—	6.5	+
<i>Staph. albus</i> .....	15	+	+	+ or —	5.8	+
<i>M. freudenreichii</i> .....	9	—	+	+	5.6	—
<i>M. ureae</i> .....	2	—	Sl.	—	6.8	—
<i>Str. liquefaciens</i> .....	2	+ (slow)	+	+	4.8	+
<i>Str. bovis</i> ...	2	+	+	—	5.6	+
<i>Achromobacter coadunatum</i> .....	7	+	+	+	5.8	+
<i>Achromobacter liquefaciens</i> .....	6	+	+	—	6.8	+
<i>Flavobacterium lactis</i> .....	1	+	+	+	6.5	+
<i>Lactis cocci</i> .....	4	—	+	+	4.8	—
<i>Alcaligines</i> ?.....	4	—	+	—	7.2	—
<i>Lactic rods</i> .....	9	—	+	+	4.6	—
<i>Coccus</i> .....	2	—	+	—	6.4	—

\* In casein media to be described in a following paper.

In table 1 is shown a comparison of the action of some of the organisms on ordinary milk agar and on the new casein agar. This table also includes the pH value which these organisms attain in milk and the ability of the organisms to decompose casein as shown by analyses to be given in a following paper. Species names have been determined only for those organisms which have been found by further test to be proteolytic in milk; hence the last three species in the table are not named. Organisms

such as *B. cereus*, *B. vulgatus*, *B. subtilis*, and *Flavobacterium synxanthum*, which are obviously proteolytic in milk or on either kind of plates, have been omitted from the table for the sake of brevity.

*M. perflavus* would be called caseolytic according to the milk agar plates, but the new casein agar plates show the organism to be non-caseolytic, and analytical data support that conclusion. *Streptococcus liquefaciens* produces a similar effect on the milk agar plates, yet is caseolytic according to both casein agar plates and chemical analyses. Organisms such as *M. casei* or *Staphylococcus albus* are apt to give a precipitate as well as a halo on the milk agar plates. When this occurs there arises a question as to whether acid or a caseolytic enzyme causes the halo. Occasional organisms, such as the unnamed "coccus" in the table, which produce little acidity, give a halo with milk agar plates but none in the new casein agar and no caseolysis in casein media. *Achromobacter liquefaciens* would be judged non-caseolytic according to the old casein agar plate method, in which the plates were flooded with acid after forty-eight hours of incubation; but this organism is distinctly positive after twenty-four hours on the new casein agar here described. Likewise some of the strains of *M. varians* would be considered doubtful on the old casein agar but are clearly positive on the new casein agar.

If lactose or other fermentable sugar were absent from milk agar plates the true digesters could be readily noted; but since the removal of lactose is impracticable, the alternative is to make a synthetic medium in which the casein is in a fine suspension similar to its condition in milk. In order to make the test more delicate only one-tenth of the amount of casein in milk was used.

The casein agar which has finally been adopted as most satisfactory for the isolation of caseolytic organisms is made as follows: 3.5 grams of casein (according to Hammarsten) are soaked for fifteen minutes in 150 cc. of distilled water and 72 cc. of saturated lime water are then added. The mixture is shaken until the casein is almost dissolved, 0.35 gram of potassium citrate is added, and the shaking is continued until the casein is dissolved, after which 10 cc. of double strength beef infusion are added and the solution is made up to 300 cc. To this solution are added



100 cc. of a 0.15 per cent calcium chloride solution and 100 cc. of a phosphate solution which contains 0.105 per cent of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (Sorensen's phosphate) and 0.035 per cent of  $\text{KH}_2\text{PO}_4$  and which has a pH value of 7.4. The resulting 500 cc. of casein solution are divided into 50 cc. portions in 200 cc. flasks and autoclaved at 20 pounds pressure for fifteen minutes. The resulting pH value should be 7.0. The medium will be markedly opalescent after sterilization. Likewise 50 cc. portions of 3 per cent washed agar are sterilized in separate flasks. When the medium is to be used the casein solution and agar are heated in the steamer and while hot the agar is poured into the flasks of casein solution and thoroughly mixed. Plates are poured with the milky solution which results. The casein or agar solutions may be steamed separately a number of times without apparent change; but after the solutions are mixed they should be used without further steaming. The mixture will withstand a few meltings but gradually becomes more flocculent and finally, on further steaming, goes into larger clots.

The action of some of the common milk organisms on the new casein agar plates is shown in plates 1 and 2.

#### REFERENCES

- AYERS, S. H. 1911 Casein media adapted to the bacterial examination of milk. 28th Ann. Rept. Bureau of Animal Industry, U. S. Dept. Agr., 225-235.
- FREUDENREICH, E. 1895 Bakteriologische Untersuchungen über den Reifungsprozess des Emmenthalerkases. Centralbl. f. Bakteriol., 2 Abt., 1, 168-179.
- HASTINGS, E. G. 1904 The action of various classes of bacteria on casein as shown by milk-agar plates. Centralbl. f. Bakteriol., 2 Abt., 12, 590-592.
- SLOBODSKA-ZAYKOWSKA, N. 1925 Über die Anwendung des Milchagars von Freudenreich bei der Untersuchung der Milchsäurebakterien. Biochem. Zschr., 159, 216-220.

#### PLATE 1

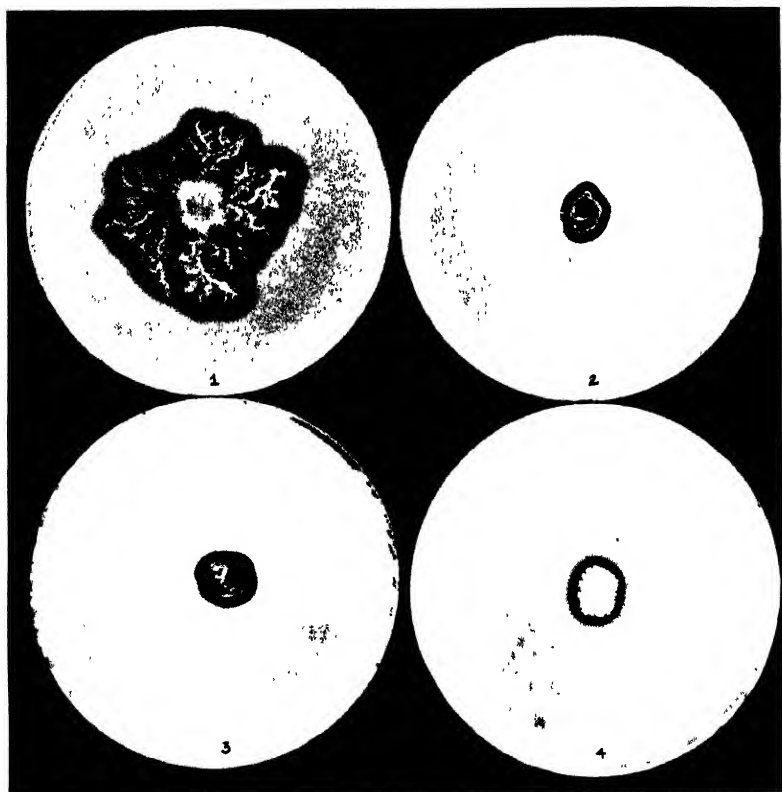
##### GIANT COLONIES ON CASEIN AGAR PLATES

FIG. 1. *B. cereus*, Strain "A."

FIG. 2. *Staphylococcus albus*.

FIG. 3. *Micrococcus citreus*.

FIG. 4. *B. cereus*, Strain "B."

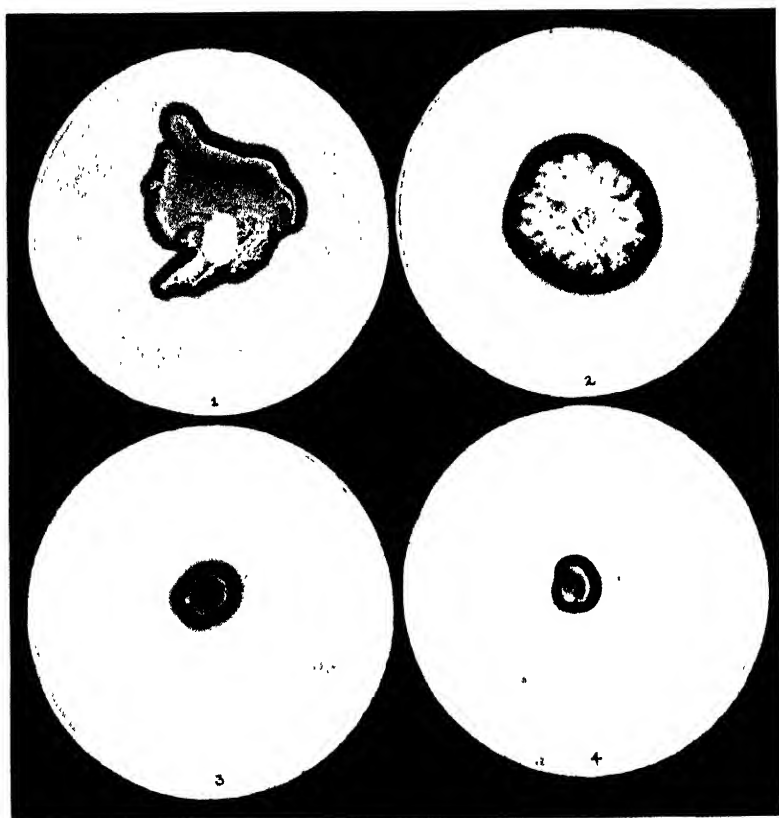


(Frazier and Rupp: Studies on proteolytic bacteria of milk.)

## PLATE 2

### GIANT COLONIES ON CASEIN AGAR PLATES

- FIG. 1. *B. vulgatus*.  
FIG. 2. *B. albolactis*.  
FIG. 3. *B. mesentericus*.  
FIG. 4. *Micrococcus casei*.



(Frazier and Rupp: Studies on proteolytic bacteria of milk.)



# STUDIES ON THE PROTEOLYTIC BACTERIA OF MILK

## II. ACTION OF PROTEOLYTIC BACTERIA OF MILK ON MILK

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In this work organisms are considered proteolytic if they can cause within a short period, a measurable increase in amino nitrogen in milk. Bacteria, such as *Streptococcus lactis*, which takes several months to produce any measurable change in the milk proteins, are not here considered proteolytic. The study has been limited to aerobic or facultative, mesophilic organisms found in milk.

It was planned to limit the methods used to those which would not be too laborious or technical for the average bacteriologist. For this reason the decomposition products from bacterial action have not been quantitatively analyzed for proteose nitrogen, peptone nitrogen, etc. Although an attempt has been made to determine the species name of each organism, this has not been deemed of great importance for it has been found that the organisms fall into general groups on the basis of their action in milk, casein solution, and gelatin solution.

Various workers have reported on the proteolytic action of bacteria in milk, but their work has been mostly confined to a study of the action of a few species or limited groups. In most cases laborious chemical analyses for decomposition products were made. Since the quantitative results obtained vary with different methods and workers the value of these results is limited.

Lisk (1924) studied the decomposition products of spore-bearing bacteria in heated milk and used *B. cereus*, *B. albolactis*, *B. megatherium*, *B. simplex*, *B. mesentericus-vulgatus*, *B. mesen-*

*tericus-fuscus*, *B. subtilis-viscosus* and *B. brevis*. She found that all these organisms caused a constant and progressive increase in ammonia and amino-nitrogen and that with all but *B. albolactis* there was a decrease in hydrogen-ion concentration accompanied by an increase in titratable acidity. Swiatopelk-Zawadski (1916) used the biuret test as a measure of proteolysis and on this basis found the following organisms proteolytic in milk: *Cl. foetidum*, *B. coli-commune*, *B. cyanogenes*, *B. lactis-Adametz*, *B. mesentericus-vulgatus*, *M. freudenreichii*, *Paraplectrum foetidum*, *B. prodigiosus*, *B. pyocyaneus*, *Staphylococcus aureus* and *B. subtilis*. There is some question as to the reliability of the biuret test as a measure of the proteolytic activity of microorganisms. Zaribnicky (1926) precipitated the casein from milk with methyl alcohol and magnesium sulphate and analyzed the filtrate for total nitrogen. By this method he found that *B. coli* did not break down the casein of milk; *B. prodigiosus* had slight action; *B. mesentericus*, *B. subtilis* and *B. pyocyaneus* were actively proteolytic; *B. mycoides* was less active; while with *Staph. pyogenes-albus* and *citreus* the action was not so rapid nor so complete. According to Taylor (1902) *B. coli* digests pure casein to proteoses and no farther. Kendall, Day and Walker (1914), who measured proteolysis by increase in ammonia, state that *B. coli* does not attack milk proteins to any appreciable extent and that *B. cloacae* has little more action than *B. coli*. They report that *B. proteus* and *B. mesentericus* are actively proteolytic and make the milk progressively more acid; *B. subtilis* produces a progressive alkaline reaction; *B. pyocyaneus* produces a transient initial acidity; and *Staphylococcus aureus* apparently produces at least slight proteolysis as measured by ammonia production. It has since been pointed out by a number of workers that ammonia production is not a reliable measure of proteolysis, especially in the presence of a fermentable sugar. The action of *B. subtilis*, *B. mycoides* and *Proteus vulgaris* on proteins was studied by Robinson and Tartar (1917) who found that all forms of nitrogen in a protein are changed to ammonia. No form is completely destroyed although mono- and di-amino nitrogen compounds are the chief sources of ammonia. These investigators report that an organism does not

act on different proteins in the same way and that bacterial action is apparently a hydrolysis carried as far as amino acids. Gorini (1925) has worked with the "acido-proteolytic" micrococci and streptococci of the cow's udder. Spitzer and Weeter (1917) studied the action of *B. proteus*, *B. liquefaciens*, *B. subtilis*, *B. megatherium*, *B. viscosus* and *B. mesentericus* in milk and found that these organisms caused a marked increase in ammonia and amide nitrogen. Waksman and Lomanitz (1925) found that *B. cereus* decomposes casein whereas *B. fluorescens* does not.

Orla-Jensen (1919) lists the following as caseolytic lactic acid bacteria: *Str. liquefaciens*, *Str. bovis*, and *Tetracoccus liquefaciens*. *Str. lactis* is called caseolytic but the action is very slow and the same is true of *Streptobacterium casei* and most organisms of his genus *Thermobacterium*.

Few attempts to classify the proteolytic bacteria of milk as such have been reported. Weigmann (1924) divides them into non-spore formers and spore formers. In the nonspore-forming class he includes the acid- and rennin-forming cocci and rods, the proteus group, the alkali-forming bacteria, and the fluorescent bacteria. Most of the spore formers belong to the earth, potato or hay bacillus group.

In some of the classifications of bacteria proteolytic activity in milk is judged entirely by the appearance of the milk cultures, but this method is not at all reliable.

#### METHODS

The organisms here studied were isolated from as many sources of milk as were available. A few of the samples of milk were incubated at temperatures of 10, 15, 25, 30, 37 and 42°C., and a few samples were pasteurized. This treatment was given to favor the development of organisms which might otherwise be missed. The first 230 organisms were picked from milk-agar plates. As was pointed out in a previous paper (1928) this method is not reliable and a new casein agar was devised from which caseolytic organisms could be picked directly. The 59 cultures picked from the new medium were all shown by further



tests to be caseolytic, whereas of the original 230 organisms from the milk-agar plates 86 were not proteolytic.

As was stated above only comparatively simple methods were used in this work. Increase in amino nitrogen was measured, ammonia was estimated roughly, and tests were made for the identification of indol, indol-acetic acid, and tryptophane. Since proteolysis is greatly influenced by acid formation, increase in acidity or alkalinity was measured by titration and by hydrogen-ion determinations.

Increase in amino nitrogen was measured by formol titration: 5 cc. of the medium were pipetted into a flask and 40 cc. of distilled water were poured through the pipette to rinse out adhering particles. This is particularly important in the presence of a casein curd. The sample was titrated with  $N/10$  NaOH to a pink color with phenolphthalein, and 5 cc. of neutralized formaldehyde solution were then added. The solution was mixed thoroughly and then titrated back to the same pink color. The number of cubic centimeters of  $N/10$  NaOH necessary was used as a measure of amino nitrogen and was expressed on the basis of 100 cc. of the medium tested. The control titration was subtracted from each culture determination to show the increase in amino nitrogen. The method of Thomas as outlined in the Manual of Methods of the Society of American Bacteriologists was followed in the estimation of ammonia. One cubic centimeter of the culture was diluted with 10 cc. of water and 1 cc. each of the phenol and sodium hypochlorite solutions were added. The intensity of the blue color produced is a rough measure of the amount of ammonia present. A test was made for indol with the Ehrlich reagent by the stopper method as well as by the test tube method, and for indol-acetic acid by Salkowski's reagent; but neither the indol nor indol-acetic acid test was found useful in milk. Of the various tests for tryptophane the bromine test has been found to be the best. Homer (1915) states that the bromine test is specific for free tryptophane. It has been suggested that tryptophane is probably one of the first compounds split from the casein molecule, and in this work it was found that the test for

free tryptophane was usually positive when there was even a small increase in amino nitrogen.

The bromine test as used in this work was carried out as follows: To 1 cc. of the milk or casein medium was added 0.5 cc. of glacial acetic acid, and the mixture was shaken until the casein had dissolved. To this solution saturated bromine water was added drop by drop with shaking. To determine the number of drops of bromine water to be used it was first added to the acidified control until a permanent yellow color appeared. Then half that number of drops was added to the solutions to be tested. In the presence of free tryptophane a reddish or violet color develops after a few minutes. This color darkens on standing.

Milk sterilized by autoclaving and milk sterilized by intermittent steaming were used as culture media. It has been stated by Gorini (1917, 1921) and others that milk is so modified by heat that the proteolytic properties of the lactic bacteria are inhibited and steamed milk is therefore preferable to autoclaved milk.

#### DATA

The 229 cultures studied have been classified as far as possible into species or at least into genera and named whenever possible according to Bergey's Manual of Determinative Bacteriology. The name in parentheses after each species name refers to the classification used in naming the organism. The classification of the micrococci by Hucker (1924) was used in naming some of the cocci which could not be named according to Bergey's Manual. The spore-forming rods were divided into species with the aid of the descriptions furnished by Ford and his collaborators (1916). A discussion of the classification of the organisms will be found in a following paper.

Of the 135 cocci the following 5 were streptococci:

	<i>Number of cultures</i>
<i>Streptococcus liquefaciens</i> Orla-Jensen (Bergey).....	3
<i>Streptococcus bovis</i> Orla-Jensen (Bergey).....	2

Of the 130 micrococci 73 cultures were classed as producing yellow pigment and 57 were non-chromogenic. The yellow cocci were divided as follows:

	Number of cultures
<i>M. citreus</i> Migula (Hucker).....	31
<i>M. perflavus</i> (Bergey) or <i>M. aureus</i> Rosenbach (Hucker).....	11
<i>M. varians</i> Conn (Bergey).....	9
<i>M. casei</i> <sup>1</sup> (Hucker) or <i>Tetracoccus liquefaciens</i> (Orla-Jensen)—yellow strains .....	7
<i>M. percitreus</i> (Bergey) or <i>B. conglomeratus</i> Migula (Hucker).....	5
<i>M. luteus</i> Cohn (Bergey).....	5
<i>M. cereus</i> Passet (Bergey).....	3
<i>M. subflavescens</i> (Bergey) or <i>M. flavus</i> Lehmann and Neumann (Hucker). 1	

The 57 non-chromogenic micrococci were divided as follows:

	Number of cultures
<i>M. casei</i> <sup>1</sup> (Hucker) or <i>Tetracoccus liquefaciens</i> (Orla-Jensen)— white strains .....	28
<i>Staph. albus</i> Rosenbach (Bergey) or <i>M. albus</i> Rosenbach (Hucker)....	15
<i>M. freudenreichii</i> Guillebeau <sup>2</sup> (Bergey).....	9
<i>M. urcae</i> Cohn (Bergey).....	2
Unidentified—P 147.....	1
Unidentified—P 204.....	1
Unidentified—P 269.....	1

Of the 94 rod-shaped organisms 37 were Gram-negative and 57 Gram-positive. The following three Gram-negative rods produced a red pigment:

	Number of cultures
<i>Serratia ruber</i> Zimmermann (Bergey).....	2
<i>Serratia indica</i> Koch (Bergey).....	1

The following 16 Gram-negative rods produced a yellow pigment:

	Number of cultures
<i>Flavobacterium synxanthum</i> Ehrenberg (Bergey).....	13
<i>Flavobacterium lactis</i> Grimm (Bergey).....	1
<i>Flavobacterium tremelloides</i> Copeland (Bergey).....	1
Unidentified—P 268.....	1

<sup>1</sup> Except did not use ammonia as a sole source of nitrogen.

<sup>2</sup> Except nitrates reduced.

The following 18 Gram-negative rods were non-chromogenic:

	<i>Number of cultures</i>
<i>Achromobacter coadunatum</i> Wright (Bergey).....	7
<i>Achromobacter liquefaciens</i> Frankland (Bergey).....	6
<i>Achromobacter delictatulum</i> Jordan (Bergey).....	1
<i>Proteus vulgaris</i> Hauser (Bergey).....	1
<i>Alcaligines bookeri</i> Booker (Bergey) .....	2
Unidentified ( <i>Escherichia</i> ) P 107.....	1

Spores were found in all but two of the Gram-positive rods neither of which could be identified. The remaining 55 Gram-positive rods are as follows:

	<i>Number of cultures</i>
<i>B. albolactis</i> Migula (Ford).....	28
<i>B. cereus</i> Frankland (Ford).....	11
<i>B. vulgaris</i> Trevisan (Ford).....	6
<i>B. subtilis</i> Cohn (Ford).....	2
<i>B. simplex</i> Gottheil (Ford).....	2
<i>B. mesentericus</i> Flügge (Ford).....	1
<i>B. cohaerens</i> Gottheil (Ford).....	1
<i>B. tumescens</i> Zopf (Bergey).....	1
<i>B. megatherium</i> De Bary (Ford).....	1
<i>B. ruminatus</i> Gottheil (Ford).....	1
<i>B. macerans</i> Schardinger (Bergey).....	1

The cultures of *B. albolactis* and *B. cereus* could be subdivided into strains which differ in several characteristics, but in this paper only *B. cereus* is divided into two strains "A" and "B."

#### ACTION OF ORGANISMS ON MILK

In tables 1, 2, and 3 are summarized the results of the action of the various organisms on milk sterilized in the autoclave and on milk sterilized by steam at 100°C. The results are expressed as averages for all the cultures within each species.

In table 1 it will be noted that 127 cocci of the total 135 bring the pH value of milk to below 6.0, that is, all of them ferment lactose and can be considered acido-proteolytic. Since the remaining 8 cultures which do not ferment lactose and do not acidify the milk can be divided into 4 species, they may be considered as occasional and probably of little importance in milk. All but

*M. subflavescens* give very little increase in amino nitrogen in milk.

The bromine test for tryptophane is positive in 104 of 135 cases where the cocci are proteolytic in milk. It will be shown in a following paper that all but 3 of the negative cultures fail to de-

TABLE 1

*Action of cocci on autoclaved and steamed milk after ten days at 30°C.*

ORGANISM	NUMBER OF CULTURES	AUTOCLAVED MILK					STEAMED MILK			
		pH	Titrateable acidity*	NH <sub>3</sub>	Br test	Amino-N†	Titrateable acidity	NH <sub>3</sub>	Br test	Amino-N
		cc.				cc.	cc.			cc.
Control.....		6.6	0.0	—	—	0.0	0.0	—	—	0.0
<i>M. citreus</i> .....	31	5.6	38.0	—	+	5.3	36.6	—	+	7.3
<i>M. perflavus</i> .....	11	5.9	41.0	Sl.	—	8.7	32.2	—	—	10.8
<i>M. varians</i> .....	9	5.4	49.2	+	+	10.8	42.0	—	+	8.0
<i>M. casei</i> (yellow).....	7	5.7	38.2	—	+	6.2	31.6	—	+	5.5
<i>M. percitreus</i> .....	5	5.3	27.8	+	—	14.6	25.9	—	—	10.9
<i>M. luteus</i> .....	5	5.4	48.4	—	+	8.6	49.3	—	Sl.	6.9
<i>M. cereus</i> .....	3	6.5	—4.2	+	—	8.2	—1.2	—	—	2.6
<i>M. subflavescens</i> .....	2	6.4	17.8	+	+	14.5	18.6	—	Sl.	10.5
<i>M. casei</i> (white).....	28	5.7	40.0	—	+	4.8	34.0	—	+	5.4
<i>Staph. albus</i> .....	15	5.8	34.9	—	+	5.9	33.2	—	+	7.9
<i>M. freudenreichii</i> .....	9	5.6	39.3	Sl.	—	6.0	32.5	—	—	6.2
<i>M. ureae</i> .....	2	6.8	—6.2	+	—	5.6	—6.6	+	—	4.2
P 147.....	1	4.7	57.6	—	Sl.	9.8	21.2	—	Sl.	6.4
P 204.....	1	4.6	89.0	—	+	5.0	92.0	—	+	5.2
P 269.....	1	6.5	22.0	—	—		20.0	—	—	1.8
<i>Str. liquefaciens</i> .....	3	4.8	99.2	+	+	10.2	86.8	—	+	7.0
<i>Str. bovis</i> .....	2	5.6	33.2	—	+	5.4	31.8	—	+	4.3

\* Increase in acidity expressed as cubic centimeters of N/10 NaOH to neutralize 100 cc. of medium to phenolphthalein.

† Increase in amino-N expressed as cubic centimeters of N/10 NaOH per 100 cc. of medium.

compose casein but break down lactalbumin and hence do not give a positive bromine test.

The Gram-negative rods in table 2 are all positive to the bromine test when they cause an increase in amino nitrogen with the exception of 2 cultures which are very weakly proteolytic. Atten-

tion must be directed to the organisms which have been called *Achromobacter coadunatum* and which are included in the study of the proteolytic organisms although the table shows no increase in amino nitrogen in milk. Of the 7 cultures of this species 3 usually give an increase in amino nitrogen and the other 4 none,

TABLE 2

*Action of Gram-negative rods on autoclaved and steamed milk after ten days at 30°C.*

ORGANISM	NUMBER OF CULTURES	AUTOCLAVED MILK					STEAMED MILK			
		pH	Titrateable acidity*	NH <sub>3</sub>	Br test	Amino-N	Titrateable acidity	NH <sub>3</sub>	Br test	Amino-N
Control.....		6.6	0 0	—	—	0.0	0 0	—	—	0.0
<i>Flavobacterium synxanthum</i> .....	13	7 0	29.7	+	++	77.2	27 5	+	++	66.2
<i>Flavobacterium lactis</i> ....	1	6 5	2.4	—	—	3 2	0 8	—	—	3.2
<i>Flavobacterium tremeloides</i> .....	1	6 2	28.2	Sl.	Sl.	10.0	26 2	Sl.	Sl.	9.4
P 268.....	1	7.0	21.3	+	+	38.0	20.0	+	+	37.8
<i>Achromobacter coadunatum</i> .....	7	5 8	27.5	Sl.	—	0.0	29.6	—	—	0.0
<i>Achromobacter liquidifaciens</i> .....	6	6 8	6.7	Sl.	+	12.5	7.2	+	+	16.8
<i>Achromobacter delictatulum</i> .....	1	7.0	28.4	Sl.	+	17.8	21.8	+	+	12.2
<i>Proteus vulgaris</i> .....	1	6.8	25.6	—	+	27.8	34.6	—	+	53 8
<i>Alcaligines bookeri</i> .....	2	8.2	11.2	Sl.	++	53.8	6.5	—	++	96 5
P 107 ( <i>Escherichia</i> ).....	1	4 8	59.6	—	Sl.	4.0	53 0	—	—	0.0
<i>Serratia ruber</i> .....	2	8.2	—14.0	+	—	3 6	—16 4	Sl.	—	3.2
<i>Serratia indica</i> .....	1	7.0	32 2	+	+	21.2	27.6	+	+	31.4

\* See footnotes to table 1.

although all 7 organisms very evidently belong together. These organisms are lactose fermenters and in the presence of lactose in milk may or may not appreciably break down the milk proteins. It will be shown in a following paper, however, that these organisms are very actively caseolytic when the amount of fermentable sugar is decreased.

The Gram-positive, spore-forming rods (table 3) fall into three groups on the basis of their action in milk:

1. The high acid, low amino-nitrogen group. These are lactose-fermenting organisms which cause a comparatively small increase in amino nitrogen in milk within ten days. *B. albolactis* is an example of this group.

TABLE 3  
Action of Gram-positive rods on autoclaved and steamed milk

ORGANISM	NUMBER OF CULTURES	AUTOCLAVED MILK							STEAMED MILK			
		After ten days at 30°C.					After 30 days					
		pH	Titratable acidity*	NH <sub>3</sub>	Br test	Amino-N	Titratable acidity	Amino-N	Titratable acidity	NH <sub>3</sub>	Br test	Amino-N
cc.				cc.	cc.	cc.	cc.				cc.	
Control.....		6.6	0.0	—	—	0.0	0.0	0.0	0.0	—	—	0.0
<i>B. albolactis</i> .....	28	5.3	70.6	Sl.	+	17.3	92.2	34.4	66.0	—	+	15.4
<i>B. cereus</i> , strain "A"	8	7.3	18.8	+	++	66.4	30.8	126.8	16.4	+	+	50.8
<i>B. cereus</i> , strain "B"	3	7.3	6.4	++	++	75.0	14.2	135.2	9.1	++	+	36.6
<i>B. vulgatus</i> .....	6	6.9	11.2	+	++	62.8	20.1	107.2	9.7	+	+	45.4
<i>B. subtilis</i> .....	2	7.4	18.6	+	++	53.4	13.7	81.1	10.3	+	+	28.9
<i>B. simplex</i> .....	2	6.5	19.2	—	+	8.7	34.4	18.9	10.4	—	—	5.7
<i>B. mesentericus</i> .....	1	6.6	19.6	Sl.	Sl.	20.2	28.2	17.6	8.6	—	—	3.6
<i>B. cohaerens</i> .....	1	6.8	12.8	Sl.	+	19.0	19.0	23.4	8.8	—	Sl.	5.4
<i>B. tumescens</i> .....	1	6.8	11.0	+	++	42.6	12.8	122.4	11.2	++	+	35.6
<i>B. megatherium</i> .....	1	7.0	22.6	—	++	28.2	35.4	92.8	17.8	—	+	24.8
<i>B. ruminatus</i> .....	1	6.4	32.8	—	+	10.4	55.0	40.6	8.6	—	—	0.0
<i>B. macerans</i> .....	1	5.5	38.8	—	+	2.8	41.8	16.6	25.0	—	Sl.	0.0
P 67.....	1	5.3	—16.0	—	—	0.0			—15.4	Sl.	—	2.2
P 285.....	1	6.5		—	—				3.6	—	—	3.4

\* See footnotes to table 1.

2. The low acid, low amino-nitrogen group. This group produces a slightly acid reaction due to protein decomposition and a comparatively small increase in amino-nitrogen. *B. mesentericus* and *B. simplex* are examples.

3. The low acid, high amino-nitrogen group. This group produces an alkaline reaction and a considerable increase in amino nitrogen. *B. cereus* and *B. vulgatus* are examples.

The organisms which have been included under the name *B. albolactis* are sufficiently alike in their action on milk to be grouped together in this discussion, and would all be called *B. albolactis* according to the descriptions which are usually given. On the basis of some of their characteristics, however, they can be still further subdivided. The two strains of *B. cereus* given in table 3 coincide in general with the descriptions of that organism, but differ between themselves in a number of characteristics.

It will be observed that in the autoclaved milk all the proteolytic rods gave a positive bromine test; but not all were positive in steamed milk. The ammonia test was less trustworthy. Al-

TABLE 4

*Weighted averages of amino-nitrogen titrations in autoclaved and steamed milk*

ORGANISMS	NUMBER OF CULTURES	AMINO-N IN AUTOCLAVED MILK*	AMINO-N IN STEAMED MILK
		cc.	cc.
All cocci.....	135	6.46	7.07
All Gram-negative rods.....	37	45.07	45.17
All Gram-positive rods.....	57	33.87	24.73
All high in amino-N†.....	75	41.41	32.89
All low in amino-N‡.....	140	6.85	7.32

\* Expressed as cc. of N/10 NaOH.

† Over 15.0 cc. of N/10 NaOH.

‡ Under 15.0 cc. of N/10 NaOH.

though the proteolytic action of the cocci is almost completed after ten days at 30°C. the action of the spore-forming rods continues and for this reason amino titrations after thirty days incubation are included in table 3.

To summarize the results with the bromine test in milk: Of the 222 cultures which gave an increase in amino nitrogen, 197 were positive to the bromine test. All but 10 of the negative cultures are non-caseolytic. It will be shown later that these 10 negative cultures are able to decompose lactalbumin as well as casein. The negative bromine test may indicate a preference for the lactalbumin in milk. That the bromine test is fairly reliable for the detection of caseolysis will be shown in a following paper.



In table 4 a comparison is made of the weighted averages of the amino titrations of the main classes of organisms in autoclaved and steamed milk. It will be observed that autoclaved milk apparently has no advantage over steamed milk for cultures which are weakly proteolytic, but that in the case of the more actively proteolytic organisms the autoclaved milk shows an increase of

TABLE 5

*Action of various stock cultures on steamed milk after ten days at 30°C.*

ORGANISM	TITRAT- ABLE ACIDITY*	NH <sub>3</sub>	Br TEST	AMINO-N
	cc.			cc.
Control.....	0.0	—	—	0.0
<i>B. simplex</i> .....	-12.2	—	—	0.0
<i>B. megatherium</i> .....	35.4	—	+	12.8
<i>B. vulgatus</i> .....	-2.0	—	Sl.	9.0
<i>B. tumescens</i> .....	-1.0	Sl.	—	0.0
<i>B. mycoides</i> .....	-1.4	Sl.	Sl.	22.4
<i>B. subtilis viscosus</i> .....	1.0	—	—	0.0
<i>B. laterosporus</i> .....	-4.8	+	Sl.	9.4
<i>B. globigii</i> .....	-1.4	—	—	-3.0
<i>B. subtilis</i> .....	18.2	+	++	32.6
<i>B. sphaericus</i> .....	-2.8	—	—	-2.6
<i>B. albolactis</i> .....	16.6	+	++	45.0
<i>B. adhaerens</i> .....	24.4	—	+	15.6
<i>B. cohaerens</i> .....	24.0	—	+	20.6
<i>B. graveolens</i> .....	9.2	—	Sl.	4.0
<i>B. niger</i> .....	21.0	—	+	11.0
<i>Proteus vulgaris</i> .....	22.0	+	+	42.2
<i>Escherichia neopolitana</i> .....	74.0	—	—	-3.2
<i>Escherichia coli</i> .....	60.4	—	—	-5.4
<i>Aerobacter aerogenes</i> .....	46.8	—	—	-12.6

\*See footnotes to table 1.

about 26 per cent in amino nitrogen over that of the steamed milk.

In table 5 are given the results of the action of a number of organisms, most of which were obtained originally from various pure culture collections and which have been carried in stock for some time. It will be noted that the amino-nitrogen results obtained are for the most part much lower with these organisms

than with the more freshly isolated cultures used in this work, although all the milk cultures were made from fresh transplants. The *B. albolactis* culture, on the other hand, seems to have lost most of its ability to ferment lactose and has become correspondingly more proteolytic. It has been stated by other workers that the proteolytic activity of cultures may change after a number of transfers in pure culture.

A study of the action of the proteolytic milk organisms on synthetic casein media and gelatin media is of assistance in their general grouping and will be discussed in a following paper.

#### SUMMARY

Two hundred and twenty-nine cultures of proteolytic milk organisms were isolated and classified, as far as possible, into species. Data are given to show the change in amino-nitrogen, titratable acidity, hydrogen-ion concentration, and ammonia; and tests were made for tryptophane in cultures in both autoclaved and steamed milk held for ten days at 30°C.

The cocci were found to be mostly acido-proteolytic and to produce only a moderate increase in amino-nitrogen in milk. The Gram-negative rods varied from weakly proteolytic to actively proteolytic. The Gram-positive spore-forming rods fell into three groups: (1) A high acid, low amino-nitrogen group, (2) a low acid, low amino-nitrogen group, and (3) a low acid, high amino-nitrogen group.

The bromine test for free tryptophane has been found helpful in the identification of the milk organisms which break down casein. The test is usually more satisfactory in autoclaved than in steamed milk.

Autoclaved milk favors the action of the more actively proteolytic organisms but has no advantage over steamed milk for the weakly proteolytic organisms.

#### REFERENCES

- FRAZIER, W. C., AND RUFF, P. 1928 Jour. Bacteriol., 16, 57.  
GORINI, C. 1917 Rend. R. Acad. d. Lincei, 26, 195-199, 223-227 (Abstr. in Abstracts Bacteriol., 5, 36, 1921).

- GORINI, C. 1921. *Le Lait*, 1, 57-62.
- GORINI, C. 1925 *Internatl. Rev. Sci. Pract. Agr. (Rome)*, Ser. 3, 1, 65-87.
- HOMER, A. 1915 *Jour. Biol. Chem.* 22, 369.
- HUCKER, G. J. 1924 N. Y. (Geneva) *Agr. Expt. Sta., Tech. Bul.* 102.
- KENDALL, A. I., DAY, A. A., AND WALKER, A. W. 1914 *Jour. Amer. Chem. Soc.* 36, 1937-1966.
- LAUBACH, C. A., RICE, J. L., AND FORD, W. W. 1916 *Jour. Bacteriol.* 1, 493-533.
- LAWRENCE, J. S., AND FORD, W. W. 1916 *Jour. Bacteriol.* 1, 273-319.
- LISK, H. 1924 *Jour. Bacteriol.* 9, 1-12.
- ORLA-JENSEN, S. 1919 *The Lactic Acid Bacteria*. Copenhagen.
- ROBINSON, R. H., AND TARTAR, H. V. 1917 *Jour. Biol. Chem.*, 30, 135-144.
- SPITZER, G., AND WEETER, H. M. 1917 *Proc. Indiana Acad. Sci.*, p. 91-96.
- SWIATOPELK-ZAWADSKI 1916 *Zschr. Nahr. u. Genuss.*, 32, 161-170.
- TAYLOR, A. E. 1902 *Zschr. physiol. Chem.*, 36, 487-492.
- WAKSMAN, S. A., AND LOMANITZ, S. 1925 *Jour. Agric. Res.* 30, 263-281.
- WEIGMANN, H. 1924 *Die Pilzkunde der Milch*. Berlin.
- ZARIBNICKY, F. 1926 *Milchwirt. Forsch.*, 3, 404, 432.

# OBSERVATIONS ON THE PHYSICAL AND BIOLOGICAL CHARACTERISTICS OF LEPTOSPIRA

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The group of spirochetes for which Noguchi (1918) proposed the generic name *Leptospira* has been assuming greater and greater importance since the first discovery by Inada and his co-workers (1916) and others (Huebner and Reiter, 1916; Uhlenhuth and Fromme, 1916) that a spirochete of this group is the cause of Weil's disease. Shortly after the discovery of *L. icterohemorrhagiae*, Noguchi (1919) cultivated a similar organism (*L. icteroides*) from cases of yellow fever in South America. At about the same time, Ido and his associates (1918) announced the cultivation of *L. hebdomadis*, the cause of Japanese seven day fever. More recently Vervoort (1922) isolated a *Leptospira* (*L. pyrogenes*) from cases of an unclassified dengue-like fever in Sumatra. In 1921 Couvy (1921) reported the presence of a *Leptospira* in cases of dengue in Syria and in the same year Whittingham (1921) reported the cultivation of a *Leptospira* from cases of phlebotomus fever in Malta. In 1922 Blanchard and Lefrou claimed to have found a *Leptospira* in certain cases of blackwater fever. Still more recently Prausnitz and Lubinski (1926) reported the presence of a *Leptospira* in blood cultures from cases of "swamp fever" ("Schlammfieber"), which occurred during the flood in Silesia.

Any one who has worked with this group of organisms, is familiar with the difficulties which beset one who studies them for the first time. The artifacts resulting from cell stroma often strikingly resemble these delicate organisms in form and sometimes even in structure; and their peculiar motion is also deceptive. The ease with which these artifacts may be mistaken for

*Leptospira* throws doubt on some of the reported association of *Leptospira* with certain specific diseases.

In view of the increasing importance of this group of organisms and of our own interest in the etiology of phlebotomus fever, which according to Whittingham is caused by a *Leptospira*, we considered it of importance to define more clearly the properties of these organisms in order to facilitate their differentiation. The *Leptospiras* possess properties characteristic of bacteria as well as of the filterable viruses. In the ease with which they produce antibodies, and in their adaptability to artificial cultivation, they closely resemble our simpler pathogenic bacteria. On the other hand their passage through the various types of filter candles makes it difficult to differentiate them from the so called filterable viruses.

Thanks to the generosity of Dr. Noguchi of the Rockefeller Institute, New York, Dr. Brown of the Welcome Bureau, London, and Professor Schüffner of the Institute of Tropical Hygiene, Amsterdam, who have supplied material, we have been enabled to make comparative studies on a large variety of strains from various sources. In addition we have cultivated several strains of water *Leptospira* from various sources in Palestine.

#### PHYSICAL PROPERTIES

*Morphology.* The group as a whole has a highly variable morphology. According to the condition of the medium and the temperature at which they are cultivated, one may get extremely short, medium or long forms or long chain-like threads composed of several elements. In serum peptone water short forms are the rule, in semisolid media the medium form prevails; while at higher temperatures ( $30^{\circ}+$ ) the long thread-like forms develop. One strain isolated by Brown from a case of Weil's disease in England grew persistently as a straight form characteristic of *L. hebdomadis*, until the culture became contaminated. In the contaminated culture the typical S and C forms appeared, but disappeared again when the culture was purified. Culturally and immunologically this strain reacted like a typical *L. icterohemorrhagiae*.

Photographs of some of the variations are shown in the plate.

The only strains that seem to have a more or less distinctive morphology are the water *Leptospiras*. These are consistently coarser and less finely spiral than the pathogenic strains studied. But among the pathogenic strains morphological differentiation seemed impossible; such differences as were observed being easily attributable to differences in the rates of growth, in the composition and reaction of the medium, etc.

*Electrical charge.* It was of interest to ascertain whether these organisms resemble bacteria or spirochetes in their electrical charge. Bacteria are as a rule negatively charged, while spirochetes and protozoa generally have a positive charge. Cataphoresis experiments were made in phosphate and borate buffer solutions. The *Leptospiras* travelled to the negative pole at pH 6.0 to 8.6 and to the positive pole at pH 9.2. In other words in mildly acid or neutral and mildly alkaline solutions they are positively charged, while in strongly alkaline solutions they become negatively charged. The tests were made by adding small amounts of active cultures grown in serum peptone water to diluted buffer solutions and observing the movement in a cataphoresis cell which could be watched under the dark field. The concentration of buffer must be kept low or else the organisms are injured.

*Effect of salinity, reaction, etc.* In the absence of colloids (serum, peptone, etc.) the *Leptospiras* are exceedingly sensitive to reaction and salinity. The results of a series of tests indicate that without a serum buffer, they die promptly in acid solutions above 6.6, but are much less sensitive to alkaline reactions. The optimum is between pH 7.2 and 8.2. There were slight variations in the sensitiveness of different strains, due probably to differences in the vigor of the culture, but the general behavior was the same. The only apparently constant difference was in the water strains which are decidedly more sensitive to acid reactions, finding their optimal conditions in alkaline substrates. Salinity is decidedly harmful. Even normal saline solutions injure the organisms. Peroxide solutions of 1:9000 kill the *Leptospiras* in a few seconds. At higher dilution different strains manifest individual differences. The English rat strain was

killed in a dilution of 1:8000 in five to ten minutes, while American and Holland rat strains were not effected by the same dilutions. The water strains were consistently more sensitive to peroxide than the pathogenic strains.

*Sedimentation.* Schüffner and Sieburgh (1926) have reported the fact that unlike the *Treponema* the *Leptospira* may be sedimented by centrifugalization at high speed. They propose this as an aid to rapid diagnosis.

We have performed a larger number of experiments with a variety of materials, and have been able to confirm fully the results of Schüffner and Sieburgh. The *Leptospiras* are not sedimented as bacteria, but a longer period at high speed will ultimately precipitate them. It was possible, without any difficulty, to find *Leptospiras* in the sediment of centrifugalized serum of infected guinea pigs when prolonged careful search failed to reveal any organisms by direct examination. Similarly *Leptospiras* could be demonstrated in the sediment of filtrates from Berkefeld candles when no organisms could be found after repeated careful examination of the uncentrifuged material.

The first important point in the procedure is to eliminate the blood cells—red and white—before the concentration of *Leptospira* is attempted. The presence of cells makes it difficult to find the *Leptospira*. Schüffner recommends three centrifugalizations: five to six minutes at 1500 revolutions, ten minutes at 1500 and finally thirty minutes at 3000. We found that two centrifugalizations suffice. First the blood is centrifugalized at a speed of 1500 for fifteen to twenty minutes, then the clear supernatant serum is transferred to another tube and centrifuged for one hour at 3000. This procedure brings about a sufficient concentration of the organism for ready detection. The number of *Leptospiras* in the sediment depends entirely on the original number present in the blood. Sometimes there would be several *Leptospiras* per field, at others only a few in an entire drop. An important detail is to decant the fluid completely and examine only the compact sediment taken up by a capillary pipette. (See table 1.)

The following protocol is typical of another type of experiment.

To a mixture of normal guinea pig blood and 1 per cent sodium citrate solution (3 cc. blood + 2 cc. citrate) were added with a capillary pipette 10 drops of serum from an infected guinea pig. Several drops of this serum from the same pipette were examined by the dark field and by staining methods and found to contain about 1 *Leptospira* in each drop. The citrated blood was centrifugalized twice, one-half hour at 1500 and one hour at 3000. In the second sediment 1 *Leptospira* was seen. In other words out of approximately 10 possible *Leptospiras* in 5 cc. blood (2 per cc.), 1 was recovered in the sediment.

Another type of experiment was performed in order to determine the ease with which these organisms are sedimented, whether all *Leptospiras* can ultimately be thrown down, and the length

TABLE 1  
*Experiments furnishing some idea of the degree of concentration*

GUINEA PIG NUMBER	DATE	TEMPERA- TURE	DIRECT EXAMINATION ONE-HALF HOUR	SERUM AFTER FIRST CENTRI- FUGATION (1500 REVOLU- TIONS FOR 20 MINUTES)	SEDIMENT AFTER SECOND CENTRIFUGA- TION (3000 REVOLU- TIONS FOR 1 HOUR)
18	25/1	38.2	0	0	0
	26	39.3	0	0	1 L.
	27	39.4	0	0	0
19	25	38.6	0	0	0
	26	40.2	0	0	13 L.
	27	40.0	0	1	20+L. (many)

L. = leptospira.

of time required to free a suspension of organisms. The following experiments are typical.

A. Five cubic centimeters defibrinated guinea pig blood rich in *Leptospira* was centrifugalized at 1500 revolutions for twenty minutes to remove the blood cells, and then at 3000. After forty-five minutes many *Leptospiras* were found both in the sediment and supernatant fluid. The clear supernatant fluid was again centrifugalized at 3000, and the process repeated. The results are given below:

After forty-five minutes: many *Leptospiras* in supernatant fluid and sediment.



After another hour: many *Leptospiras* in the sediment; none in the supernatant fluid.

After one hour more: Twenty to thirty *Leptospiras* in the sediment; none in the supernatant fluid.

*After one hour more:* 7 *Leptospiras* in the sediment; none in the supernatant fluid.

Another type of experiment gave similar results:

B. Nine cubic centimeters defibrinated guinea pig blood from an infected animal were divided into 3 parts:

Part a. Centrifugalized at 3000 for two hours. One cubic centimeter of the supernatant serum was injected into a normal guinea pig. The animal developed a typical infection after an incubation period of four days; with jaundice and *Leptospira* in the blood.

Part b. Centrifugalized for four hours at 3000 and 1 cc. of the supernatant serum injected into a normal guinea pig. The animal developed slight temperature after twelve days, but no *Leptospira* could be detected. Blood inoculated into other animals failed to produce an infection. The first guinea pig died subsequently from an intercurrent infection (paratyphoid).

Part c. Control. 1 cc. of the blood which was standing in the laboratory for seven hours was injected into a normal guinea pig. The animal developed a typical infection after an incubation period of three days.

Other experiments gave the same results. *Leptospiras* are evidently readily thrown down by prolonged centrifugation. But the process is a progressive one and requires a considerable amount of time for completion. It is possible to affect practically complete sterilization of a moderately infected blood in four to five hours continuous centrifugation at 3000 revolutions.

The *Leptospiras* differ in this respect so essentially from most true filterable viruses, that this characteristic may serve as a valuable means of differentiation between virus and leptospiral diseases.

*Filterability.* Since filterability through porous candles is one of the outstanding characters of the group, this property was studied in some detail. A number of experiments were made, using different types of Berkefeld filters as well as Seitz plates with

various kinds of suspensions at various pH values. The results showed consistently that while the organisms pass more or less readily through the filters, they are not really filterable in the sense applied to the filterable viruses. Their positive charge and extreme motility and flexibility makes it possible for a few of them to pass through the pores of the filters. However, even when starting with rich active cultures, the number of organisms which passed through the filters was so small that none could be found in the filtrate by a careful search under the dark field. Only after prolonged centrifugalization at high speed could *Leptospira* be demonstrated in the sediments of some of the filtrates.

The condition of the filter and the reaction and composition of the substrate, exert an influence on the filterability. The most satisfactory filtration experiments were made with serum peptone-water cultures. The usual procedure was to dilute the culture with 10 parts peptone-water before filtration. The *Leptospira* passed through the Seitz filter as well as through N and W Berkefeld candles, at reactions ranging between pH 5.8 and pH 8.6, the acid reactions being the more favorable. Cultures of *B. coli* or of some of the water bacteria were usually mixed with the leptospira cultures to determine the permeability of the filter for bacteria. The filtrates were tested both by centrifugation and culture. Filtrates of infected serum were also tested by inoculation into guinea pigs.

In all filtration experiments there was a marked diminution in the number of organisms in the filtrate as compared with the original suspension. It is difficult to estimate the decrease, but in all cases it was a hundred fold or more. The following experiments are typical:

A. One and one-half cubic centimeters of a rich culture of *L. icteroides* was diluted with 25 cc. of distilled water and filtered through a Berkefeld N candle. Five cubic centimeters of the filtrate were centrifugalized at 3000 revolutions for 1 hour. Only one *Leptospira* was found in the sediment.

B. In another experiment a contaminated culture of *L. icteroides* was diluted as above and filtered through a Seitz filter. No *Leptospiras* were

found either in the filtrate or sediment. After one month, two out of five semisolid cultures inoculated with 1.0 cc. of the filtrate showed a sparse growth.

C. Four cubic centimeters of serum from an infected guinea pig (*L. icteroides*) were diluted seven times with peptone-water citrate and filtered through an N candle. Three cubic centimeters of the filtrate were centrifugalized at 3000 one hour. One *Leptospira* was found in the sediment. One cubic centimeter of the filtrate (0.3 cc. of original blood) was inoculated into a small guinea pig. No infection developed: passage to second guinea pig resulted negatively and reinfection to test immunity resulted in the typical disease. The strain was sufficiently virulent so that 0.05 cc. of the blood produced an infection.

D. Blood of a heavily infected guinea pig was defibrinated and centrifugalized at 1500 for ten minutes. Five cubic centimeters supernatant serum was diluted 5 times with distilled water. One part filtered through a W Berkefeld candle. Filtrate (5 cc.) divided into three parts; 2 cc. cultured; 2.5 cc. sedimented at 3000, one hour; 0.5 cc. inoculated into guinea pig 39.

The other part was filtered through an N candle. Of the resulting 8 cc.: 3 cc. was cultured, 0.5 cc. was injected into a guinea pig and 4.5 was centrifugalized at 3000 for one hour.

In both fractions no *Leptospiras* were found in the sediment and no apparent infection developed in the guinea pigs. Subsequent reinfection proved absence of immunity.

E. Six cubic centimeters peptone water culture of a water strain were mixed with 90 cc. peptone-water and 30 cc. broth culture of *B. coli*, and divided into three parts:

1. Buffered at pH 5.8 and filtered through N candle with 30 mm. pressure. Resulting fluid (pH 5.8) distributed into empty test tubes as well as into tubes containing serum peptone-water. Five cubic centimeters centrifugalized at 3000 for one hour—8 *Leptospiras* in the sediment. The cultures showed no coli contamination, and *Leptospiras* were present in 4 out of 6 cultures.

2. Buffered at pH 8.6, filtered through N candle. Resulting fluid (pH 8.6) distributed as above. Five cubic centimeters centrifugalized as above. One *Leptospira* found in the sediment. The cultures were all positive after five days' incubation.

3. Buffered at 7.0 and filtered through N candle under pressure as above. Resulting fluid distributed as above. Five cubic centimeters centrifugalized at 3000 for one hour. No *Leptospira* seen in sediment. Four of the 6 cultures contained *Leptospira* after five days.

F. Six cubic centimeters rich serum peptone-water culture of *L. icteroides* mixed with 40 cc. peptone-water, and 0.5 cc. broth culture of *B. coli*. The mixture was divided into two parts:

1. Buffered at 5.8 and filtered through N candle under pressure of 30 mm.; resulting pH 6.6; 5 cc. centrifugalized at 3000 for one hour, 14 *Leptospiras* found in the sediment; none found by direct examination.

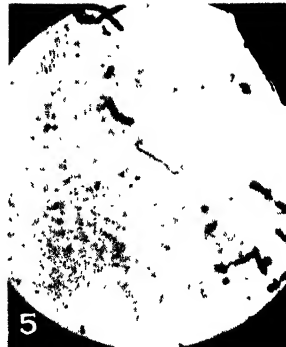
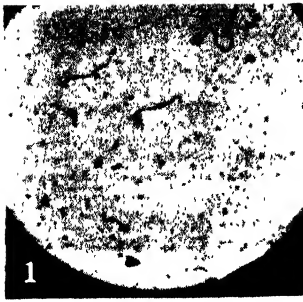
2. Buffered at 7.0 and filtered as above. Five cubic centimeters of filtrate (pH 7.2) centrifugalized as above; 18 *Leptospiras* found in the sediment.

These and other experiments indicate that *Leptospira* will pass through the standard Berkefeld and Seitz filters, but that the filtration is far from quantitative. It is questionable whether it is correct to consider *Leptospiras* as true filterable organisms. Some of these actively motile organisms go through the pores of the filters but in comparison with the amount filtered, the number which pass through is exceedingly small. In our filtration test with water *Leptospira* we obtained a pure culture of a very actively motile coarse water spirillum which like the *Leptospira* passed through the pores of the filter that held back all the water bacteria.

In this connection it is of interest to consider the character of the filtered organism. Balfour, Fantham and others have suggested that the spirochetes of relapsing fever pass through a granular stage. Noguchi reports observations which suggest the possibility of a similar occurrence in *Leptospira*. It appears from our observations on the filterability of these organisms that the assumption of a granular state is not warranted. The organisms pass the filters as *Leptospira*. They are present in the filtrate in such small numbers that they cannot be found by the ordinary procedure. If present in sufficient numbers however, centrifugation for 1 to 2 hours at 3000 revolutions will readily demonstrate typical, active, *Leptospiras* in the sediment.

#### BIOLOGICAL PROPERTIES

*Cultivation.* It is a matter of common experience, that the first cultivation of pathogenic *Leptospira* from human or animal sources is associated with considerable difficulty. Although a



FIGS. 1 TO 5

All photographs were taken with the Zeiss apparatus, ocular No. 10 oil immersion 90. Preparations stained with Fontana.

FIG. 1. *L. icteroides*—young culture in semi-solid lepto medium.

FIG. 2. *L. icterohemorrhagiae*—"hebdomadis" form. Young culture on lepto medium.

FIG. 3. *L. icterohemorrhagiae*—rat strain; thread-like form from culture grown at 30°C.

FIG. 4. *L. icteroides*—in infected guinea pig. Contrast difference in structure with leptospira from water.

FIG. 5. Water strain in water. Note coarse, heavy beading in contrast with the delicate structure of the pathogenic form.

number of satisfactory media exist for growing these organisms, primary cultivation is not uniformly successful. Even animals experimentally infected with *L. icteroides* or *L. icterohemorrhagiae* do not always yield positive cultures in consecutive trials.

Our efforts have been directed, therefore, to the question of primary cultivation of *Leptospira* from infected animals. Guinea pigs were infected with strains of *L. icteroides* and *L. icterohemorrhagiae* respectively and at various intervals after the infection cultures were made from different portions of the blood under different conditions.

The results of the various experiments were quite uniform. It appears that positive cultures can be obtained with considerable regularity by covering the fibrin clot from defibrinated blood or small amounts of sedimented red cells with Noguchi's *Leptospira* medium. For first cultures *Leptospira* medium made with saline seems much more satisfactory than the same medium made with distilled water. Subsequent subcultures grow equally well in either. In the early stages of the infection, positive cultures may also be obtained although less regularly from the clear serum inoculated into *Leptospira* medium. Citrated plasma, however, rarely gives satisfactory results.

The procedure employed consisted in drawing 2 to 5 cc. of blood from the heart of an infected guinea pig. The blood was divided into two parts. One part was distributed into tubes containing glass beads and the rest into a centrifuge tube containing citrate solution. The first fraction was defibrinated and the blood removed, leaving the clots behind. These clots were covered with Noguchi's *Leptospira* media. The defibrinated and citrated bloods were centrifugalized and the serum, plasma and sedimented cells cultured separately in various media. All cultures were incubated at 28°C.

A few typical experiments are shown in table 2.

It is needless to give more protocols. In practically every case and at various stages of the infection it was possible to cultivate the *Leptospira* if the fibrin clot or sedimented red cells were used, provided the culture was not contaminated. Whole blood as well as clear serum gave irregular results; and citrated plasma was

TABLE 2

*Experiment A. Guinea pig infected with L. icteroides*

Temperature 40°C. Direct dark field examination of the blood negative.  
Culture made November 4.

	MEDIA AND MATERIAL CULTURED	RESULTS	
		November 15	November 26
1	H <sub>2</sub> O leptospira + 3 drops of whole blood	Negative	Negative
2	Saline leptospira + 3 drops of whole blood	+ Active culture	++++ Very rich
3	0.4 per cent saline leptospira + 3 drops of whole blood	+ Active culture	++++ Very rich
4	Fibrin clot covered with 2 cc. No. 3	++++ Rich culture	++++ Very rich
5	Fibrin clot covered with H <sub>2</sub> O leptospira	+ Active culture	± Rare
6	Saline leptospira + 1 cc. plasma	± Only few	—
7	Sedimented defibrinated blood cells + saline leptospira	+++ Rich culture	++++ Very rich
8	Sedimented citrated blood cells + saline-leptospira medium	+++ Rich culture	++++ Very rich

*Experiment C. Guinea Pig infected with L. icteroides*

Temperature 39.6°C. Direct dark field examination negative.

	MEDIA AND MATERIAL CULTURED	RESULTS	
		12 days	24 days
1	H <sub>2</sub> O leptospira + 0.25 cc. serum	± Few forms	+++ Rich culture
2	Saline leptospira + 0.25 cc. serum	+++ Rich culture	+++ Rich culture
3	H <sub>2</sub> O leptospira + 0.25 cc. plasma	— No organism found	± Few inactive forms
4	Saline leptospira + 0.25 cc. plasma	— No organism found	— No leptospira seen
5	Fibrin clot in flask covered with saline leptospira medium	+++ Rich culture	+++ Rich culture

TABLE 2—Continued

*Experiment D. Guinea pig infected with L. icteroides*

Temperature 39.4°C. Dark field examination negative.

	MEDIA AND MATERIAL CULTURED	RESULTS			
		2 days	3 days	5 days	10 days
1	Clot covered with H <sub>2</sub> O leptospira media	—	±	+++	+++
2	Clot covered with saline leptospira media	—	±	+++	+++
3	Sedimented cells covered with saline leptospira media	—	—	Mold	
4	Saline leptospira + serum	—	—	Mold	

*Experiment E.*

In this experiment cultures were made from the same guinea pig on three different days, the third, fourth and ninth day after the infection. (Temperatures 39.8°, 39.6°, and 39.0°C. respectively). Positive cultures were obtained each time.

	MEDIUM + MATERIAL INOCULATED	RESULTS		
		First culture	Second culture	Third culture
1	Fibrin clot + saline leptospira media	Cont.	Cont.	+++
2	Defibrinated blood + saline leptospira media	Cont.	±	Cont.
3	Serum + saline leptospira media	Cont.	—	—
4	Sedimented cells + saline leptospira media	+++	+++	+++

most unsatisfactory. The reason for failure to cultivate *Leptospira* from plasma may be either an injurious action of citrate or the presence in the blood of substance harming the *Leptospira*. The first possibility seems the more likely because the same results were obtained in cultures during the early stages of infection as later on. Even moderate concentrations of citrate (0.5 to 1.0 per cent) exert a harmful effect on *Leptospira*, as may readily be observed by adding active cultures to normal citrated rabbit blood.

For primary cultures saline *Leptospira* media are preferable to the same media made with water. This is most probably due to an excessive liberation of hemoglobin due to the laking of the red cells. One-half per cent saline appears to be even better than the usual normal saline. Serum cultures when positive grow equally well in water media, indicating that it is not the low salinity of the medium, but the laking of the blood that is the probable injurious factor.



Most prompt and constant growth was obtained with the fibrin clot. The fibrin apparently enmeshes large numbers of organisms thus assuring a greater concentration of *Leptospira*. The same is true to a lesser extent of the sedimented cells. The sedimented red cells apparently carry the *Leptospira* with them mechanically. It is also possible that the reduction of antibodies resulting from the removal of serum is a factor. The presence of even a small amount of antibody may so greatly increase the lag as to inhibit the development of the *Leptospira* in a new substrate.

*Cultivation of water leptospira.* *Leptospiras* were found widely distributed in various parts of the country and in different kinds of water. In Jerusalem *Leptospiras* were found in a rain water pool, in cemented cisterns, in tap water, in a cement pool and in an aquarium. In the first they were demonstrated only after enrichment, while in the last they were found by direct examination. They were found in Jericho among the algae near a spring, while in Petach-Tikvah they were found in the algae in an irrigation reservoir.

We have tried several methods of cultivation.

Filtration through a Berkefeld is the obvious method, but it is not always easy to separate by this means the *Leptospiras* from other water organisms. In our experience actively motile water spirilli or spirochetes have come through the filters as readily as *Leptospiras*. Good results were obtained by primary enrichment with sterile feces according to the method proposed by Hindle, with later separation by fractional centrifugation. The method of fractional centrifugation is also useful for separating contaminated cultures of *Leptospira*. The material is suspended in water and centrifugized at 2000 revolutions for one hour. The non-motile as well as moderately motile bacteria, are thrown down while the actively motile *Leptospiras* remain in the supernatant fluid. By a series of successive dilutions of the supernatant material in serum-peptone-water pure cultures of *Leptospiras* can be obtained. We have isolated two strains of water *Leptospiras* by this method without any difficulty, and also purified some of our stock cultures which had become contaminated.

*Oxygen requirements.* All strains of *Leptospira* are obligatory aerobes. However, an interesting difference has been observed between the water *Leptospiras* and those from human and animal origin. If cultures of the various organisms are inoculated into the same media and incubated under anaerobic conditions (in Smillie jars) for various lengths of time, the pathogenic *Leptospiras* die promptly, while the water *Leptospiras* undergo an initial multiplication which soon ceases. On removal from the jar after three to ten days, no pathogenic *Leptospiras* develop whereas the water organisms resume active growth within a few days. Evidently there were pronounced differences in sensitiveness to anaerobic conditions. This distinction was quite sharp and constant with the strains under observation and by means of it the water strains studied could be readily differentiated from those of human or animal origin.

The following is a typical experiment:

Duplicates of young and old cultures of fourteen strains of *Leptospira* in semisolid and serum peptone water media were divided into three jars, two anaerobic and one aerobic. After six days one of the anaerobic jars was opened and after fourteen days the other anaerobic jar was opened.

In the first jar all tubes showed both dead and living forms, but after ten days under aerobic conditions, only the water *Leptospira* cultures were alive.

In the second anaerobic jar all cultures were apparently dead except one water strain. After ten days at room temperature all three water strains contained active *Leptospiras* while the rat and human strains were all dead.

*Pathogenic properties.* The pathogenic properties of the strains varied considerably. Organisms which have lost their pathogenicity may regain it after a series of animal passages. However, despite prolonged cultivation of the water strains in serum and blood semisolid media, they remained constant in their growth characteristics and lack of pathogenic properties. It has not been possible thus far to confirm the results reported by Uhlenhuth and Zuelzer (1921) who apparently succeeded in

transforming non-pathogenic water strains into pathogenic forms by prolonged cultivation in serum media. The three water strains studied by us, two isolated here and one obtained from Brown, have retained their cultural, serologic and saprophytic characters. The results reported by Uhlenhuth and Zuelzer and more recently by Baerman may refer to avirulent pathogenic strains which have gotten into the water.

#### DISCUSSION

In recent years the etiological rôle of *Leptospira* in the causation of certain virus diseases has assumed greater and greater prominence. Analogy plays a prominent part in scientific investigation. The reported cultivation by Noguchi (1919) of a *Leptospira* from cases of yellow fever naturally suggested that a similar organism might be the cause of clinically similar diseases such as dengue and phlebotomus fever. The presence of *Leptospira* was in fact reported by Couvy (1921) in dengue fever and by Whittingham (1921) in sandfly or phlebotomus fever. Siler, Hall and Hitchens (1926) failed to confirm Couvy's work.

It is evident that a better knowledge of the physical and biological characteristics of the *Leptospiras* may afford a basis for the study of the unknown viruses of the above named diseases. If one could establish certain constant and readily identifiable characteristics of the *Leptospiras*, these might constitute the desired criteria for such comparison. The results of the observations recorded above indicate that the sedimentability, limited filterability and ready cultivation might serve this purpose satisfactorily. Sensitiveness to the reaction of the medium, to the presence of peroxide or citrate and to variations in oxygen tension, are additional points of value.

As we have shown *Leptospiras* are readily sedimented by high speed centrifugation and if this is continued for a long time, the virulence of the supernatant serum may be greatly diminished or completely abolished. While *Leptospiras* are filter passers, the filterability is not quantitative and only a relatively small number of organisms pass through. Both of these properties sharply differentiate *Leptospira* from some of the true filter

passers which cannot be sedimented and which pass readily and apparently quantitatively through porous filters. The fact that *Leptospira* can be readily cultivated furnishes another important means of establishing similarity or difference between this organism and the viruses of presumably related diseases.

Another important consideration lies in the fact that *Leptospiras* as a group are pathogenic for guinea pigs. The pathogenicity may vary with different strains or may apparently be lost, but by repeated animal passage the virulence may be enhanced.

These properties taken together should help to ascertain whether sandfly and dengue fever are due to *Leptospira*. These criteria have actually been used in our study of the etiology of these diseases the preliminary results of which will be reported in another paper.

#### SUMMARY

The results of a comparative study of *Leptospira* from various sources may be briefly summarized as follows:

Morphologically, *Leptospiras* are exceedingly variable, the form and size depending on the medium, temperature and rate of growth.

These organisms carry a positive charge at ordinary reactions, but at pH 9.2 they become negatively charged.

The important distinguishing characters which differentiate *Leptospira* from some of the true viruses, are sedimentability and filterability. *Leptospira* may be sedimented by prolonged centrifugation at 3000 revolutions. Four to five hours seem to suffice practically to free a suspension of these organisms. Although they pass readily through a Berkefeld or Seitz filter, only a relatively small proportion of the organisms pass through.

The *Leptospiras* are readily cultivable on artificial media. The most satisfactory procedure for primary cultivation from infected animals, is to cover the blood free fibrin clot, or the sedimented red cells, with a small amount of Noguchi's *leptospira* medium (to a height of 1.5 to 2.0 cm.).

The *Leptospiras* are obligatory aerobes and the pathogenic forms die promptly when placed under anaerobic conditions.

## REFERENCES

- BLANCHARD AND LEFROU 1922 *Bul. Soc. Path. Exot.*, **15**, 699.  
COUVY 1921 *Bull. Soc. Path. Exot.*, **14**, 447.  
HUEBNER AND REITER 1916 *Deut. Med. Woch.* **42**, 1.  
IDO, Y., ITO, H., AND WAIN, H. 1918 *Jour. Exp. Med.*, **28**, 435.  
INADA, R., IDO, Y., HOKI, R., KANEKO, R., AND ITO, H. 1916 *Jour. Exp. Med.*, **23**, 377.  
NOGUCHI, H. 1918 *Jour. Exp. Med.*, **27**, 575.  
NOGUCHI, H. 1919 *Jour. Exp. Med.*, **30**, 13.  
PRAUSNITZ, C., AND LUBINSKI 1926 *Klin. Woch.*, **44**.  
SCHÜFFNER, W., AND SIEBURGH, G. 1926 *Münch. Med. Woch.*, No. 47, 1977.  
SILER, J. F., HALL, M. W., AND HITCHENS, A. P. 1926 *Monograph No. 20*,  
Bureau of Science, Manila.  
UHLENHUTH AND FROMME 1916 *Ber. Klin. Woch.*, **53**, 269.  
UHLENHUTH AND ZÜLZER 1921 *Centralbl. f. Bakt., Orig., Abt. 1*, **85**, 141.  
VERVOORT, H. 1922 *Gen. Tijds. Med. Indie*, No. 6.  
WHITTINGHAM, H. E. 1921 *Trans. Roy. Soc. Trop. Med. and Hyg.*, **15**, 149.

# THE ACTION OF IRON AND CITRATE IN SYNTHETIC MEDIA FOR TUBERCLE BACILLI<sup>1</sup>

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Several recent contributions have emphasized the importance of iron in the growth and metabolism of tubercle bacilli and suggested the value of a more detailed study of the problem. Henley (1925) has shown that the addition of small amounts of soluble iron salts to a beef infusion, peptone, glycerol broth slightly retards the initial stage but greatly promotes the later growth of tubercle bacilli. The addition of 20 mgm. of ferric sulphate per 100 cc. of media increased by 41 per cent the weight of pellicle formed in twelve weeks. Several French investigators, especially Santon (1912), working with synthetic media, have found that the addition of iron salts is favourable to the growth of the bacillus. Frouin and Guillaume (1924) reported that the presence of iron produced a favourable action upon the growth of tubercle bacilli except where the medium was very rich in nitrogen compounds and mineral salts. Long and Seibert (1926) found that the addition of ferric ammonium citrate to their synthetic medium, in the proportion of 1 to 20,000, increased the weight of pellicle formed by approximately 7.5 per cent.

It seems apparent from the literature that in many cases more stress has been laid upon the presence or absence of iron in the cultural medium than upon its availability to the growing bacilli. This latter phase of the problem is the principal consideration of this paper.

<sup>1</sup> Part of an investigation aided financially by the Canadian National Research Council Associate Committee on Tuberculosis.

# 1. INFLUENCE OF pH ON THE SOLUBILITY OF IRON IN SYNTHETIC MEDIA

A very simple synthetic medium has been used for some time in this laboratory consisting of:

Asparagin.....	4 grams
Sodium chloride.....	5 grams
Disodium phosphate.....	2 grams
Glycerol.....	30 grams
Water.....	1000 cc.

TABLE 1

*The influence of pH and citrate on the precipitation of iron in synthetic media*

pH	AMOUNT OF Na CITRATE ADDED	Fe ADDED	Fe LEFT IN SOLUTION	NATURE OF PRECIPITATE
	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	
5.8	None	0.058	0.0008	Heavy white precipitate
6.0	None	0.056	0.0010	Heavy white precipitate
6.4	None	0.055	0.0020	Brown precipitate
7.0	None	0.054	0.0034	Brown precipitate
7.4	None	0.056	0.0035	Brown precipitate
8.0	None	0.058	0.0030	Brown precipitate
4.6	0.1	0.20	0.003	White precipitate after our days
5.8	0.1	0.20	0.028	White precipitate after four days
6.0	0.1	0.20	0.018	Slight precipitate
6.2	0.1	0.20	0.200	No precipitate
6.6	0.1	0.20	0.200	No precipitate
7.0	0.1	0.20	0.200	No precipitate
7.4	0.1	0.20	0.200	No precipitate
8.0	0.1	0.20	0.180	Slight precipitate
8.4	0.1	0.20	0.0003	Brown precipitate
8.6	0.1	0.20	0.0001	Brown precipitate

In the above experiment the solutions were not autoclaved after the addition of the iron.

Several strains of tubercle bacilli and other acid-fast organisms, which have been tested, grow rapidly on this medium. The addition of inorganic iron salts, even in traces, results in precipitation of iron. From the behavior of pure solutions of iron compounds it was anticipated that the precipitation would be

influenced by the pH. The medium in several lots was therefore adjusted with NaOH and HCl to give a final pH range of 5.8 to 8.0, approximately the growth range of tubercle bacilli, and dilute solutions of ferric chloride were added in sufficient amounts to give 0.05 gram of iron per liter. Henley (1925) found the most favorable concentration of iron for the growth of tubercle bacilli to be from 0.1 to 0.3 gram of ferric sulphate per liter. The precipitate which formed after standing for 24 hours was removed by filtration and the clear solution analyzed for iron according to the method of Marriott and Wolf (1905-1906). The results are shown in table 1. It is apparent that throughout this pH range the extent of the iron precipitation is approximately equal but, what is equally significant as is shown in section 3, is that the precipitation is not quite complete, a trace of iron always remaining in the solution. The precipitate which formed in the more acid solution consisted largely of white iron phosphates while those in the solutions from pH 7.0 to a more alkaline range consisted largely of brown hydroxide of iron.

## II. INFLUENCE OF ORGANIC ACIDS ON THE PRECIPITATION OF IRON FROM SYNTHETIC MEDIA

Recently it has been shown by Reed and Haas (1924) that on the addition of sodium citrate to an inorganic nutrient solution abundant iron is held in solution for the growth of certain higher plants. Hopkins and Wann (1925) have also shown that the addition of citric acid or sodium citrate, and to a less degree of other fatty acids or their salts, to inorganic nutrient solutions (for algae) inhibits the precipitation of iron.

To the media described in the previous section, sodium citrate was added in the proportion of 0.1 gram per liter. These were adjusted to give a final pH range of 4.6 to 8.6, as used in the citrate free media and a dilute solution of ferric chloride was added in sufficient amounts to make 0.2 gram of iron per liter. After standing for twenty-four hours the media were filtered, aliquots removed and analyzed for iron as in the former case. The results are shown in table 1 along with the results of similar treatment of the citrate free media. It is apparent that the presence of a very



small concentration of citrate in this medium almost entirely inhibits iron precipitation. The inhibition of precipitation it will be observed, however, is most effective in the pH range from 6.0 to 8.0, the ordinary growth range of tubercle bacilli. In more acid and more alkaline solutions the presence of the citrate has relatively little influence on the iron precipitation.

In order to determine the possible influence of other constituents of the medium upon the solubility of iron the complete synthetic medium, as previously described, with and without citrate, was prepared and compared with a series of solutions including the ingredients of the complete medium, with glycerol,

TABLE 2

*The effects of the various constituents of a synthetic medium upon the precipitation of iron in the presence of 0.1 gram of sodium citrate per litre and in its absence*

Iron 0.2 gram as ferric chloride added to all solutions. All at pH 7.4.

CONSTITUENTS OF MEDIUM INCLUDED	CONSTITUENTS OMITTED	IRON LEFT IN SOLUTION	
		Citrate present	Citrate absent
		mgm. per cc.	mgm. per cc.
Glycerol, NaCl, phosphate, asparagin, iron.....	None	0.2	0.005
Phosphate, NaCl, asparagin, iron.....	Glycerol	0.2	0.09
Glycerol, NaCl, phosphate, iron.....	Asparagin	0.17	0.003
Glycerol, NaCl, asparagin, iron.....	Phosphate	0.2	0.002
Phosphate, NaCl, iron.....	Asparagin glycerol	0.18	0.0005

asparagin, phosphate and both glycerol and asparagin omitted. These solutions were adjusted to give a final pH 7.4, the same amount of iron was added to each. 0.2 gram per liter, as ferric chloride, and to half of each solution sodium citrate was added, 0.1 gram per liter, while the other half of each solution was left citrate-free. The results in table 2 indicate that in the absence of asparagin from the medium the citrate was very slightly less efficient in maintaining the iron in solution but that the presence or absence of the other constituents had no influence at pH 7.4.

A few other salts of iron, particularly ferric sulphate, have been tried in this medium in the presence and absence of citrate with results which are in every way comparable with those obtained

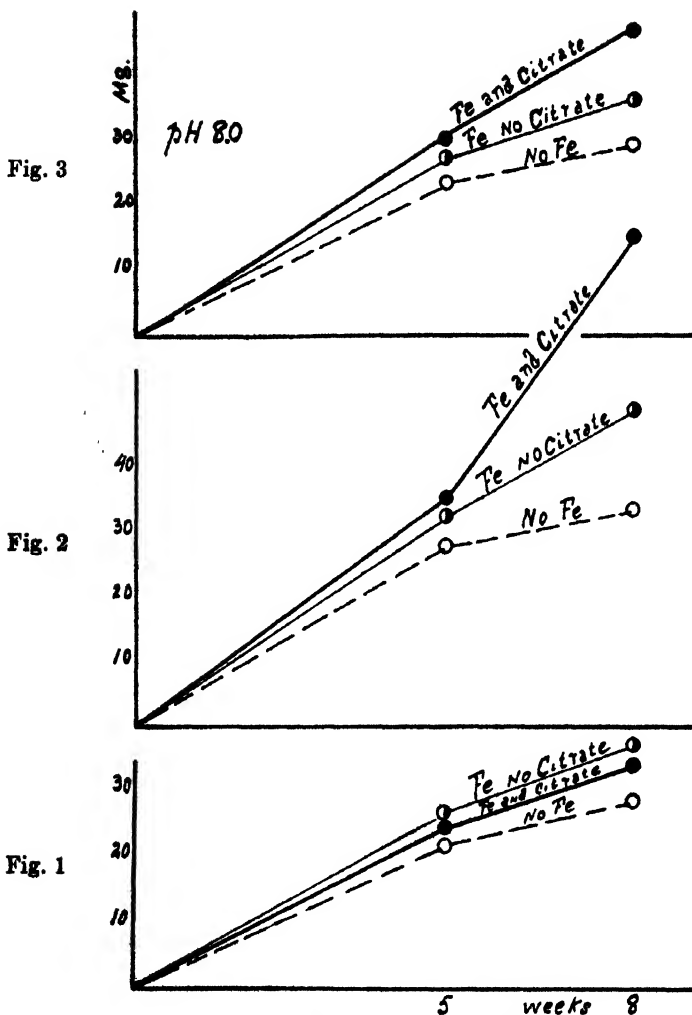
with ferric chloride. When in place of an inorganic iron salt and sodium citrate ferric citrate is added, in a proportionate amount, precisely the same effects are observed.

No attempt was made to determine experimentally the cause of the stabilizing action of citrate. It might be considered as a protective colloidal phenomenon but a much more likely explanation is the formation of "complex ions." This raises the question of the availability of iron in the complex ion but since the complex ion will always be in equilibrium with simple ions it need not influence the present consideration (cf. Stieglitz, Theoretical Qualitative Chemical Analysis).

### III. THE GROWTH OF THE TUBERCLE BACILLUS IN SYNTHETIC MEDIA WITH AND WITHOUT IRON AND WITH AND WITHOUT CITRATE

In order to test the effect of adding iron and of maintaining the iron in solution on the growth of the tubercle bacilli in a synthetic medium, media were prepared as described in the two previous sections and divided into three series, to contain in addition to the asparagin, glycerol, phosphates, and chloride (a) citrate and iron (b) iron but no citrate (c) citrate but no iron. The citrate, as in the former case, consisted in 0.1 gram sodium citrate per liter and the iron 0.11 gram per liter as ferric chloride. Each series was divided into five portions and adjusted to pH 6.0, 6.5, 7.0, 7.4 and 8.0, respectively. The series with the iron and citrate and with iron and no citrate exactly correspond with those described in Sections I and II, the iron content of which is shown in table 1, except that only approximately half the amount of iron was added and that these solutions were autoclaved. Results of analysis for iron both before and after growth of tubercle bacilli are shown in Section IV.

These preparations in 100 cc. amounts were placed in 250 cc. Erlenmeyer flasks, eight flasks of each preparation. The Koch strain of human tubercle bacilli, was used as a test organism. This is a moderately rapidly growing strain, originally obtained from the Lister Institute but has been grown in this laboratory for some years.



FIGS. 1, 2 AND 3. CURVES SHOWING THE INFLUENCE OF THE ADDITION OF IRON AND OF IRON AND CITRATE TO SYNTHETIC MEDIA UPON THE GROWTH OF TUBERCLE BACILLI

The data are taken from table 3. Ordinates represent weight of growth, abscissae time in weeks. Heavy lines, —●—, growth in media containing iron and citrate; light lines, —●—, in media containing iron and no citrate; dash lines, —○—, in media containing citrate but no iron. Figure 1 (below), growth at pH 6.25, average of data for pH 6.0 and 6.5; figure 2 (middle), growth at pH 7.2 average of data for pH 7.0 and 7.4; figure 3 (above), growth at pH 8.0.

The amount of growth was determined quantitatively after five and eight weeks incubation. The cultures to be examined were autoclaved at 15 pounds for twenty minutes, filtered through weighed Gooch crucibles containing a layer of filter paper, the organisms on the filter washed with a considerable volume of water, dried at 90° to constant weight, about twelve hours, and weighed. The results recorded in table 3 represent the average of two series of experiments. To facilitate comparison these data have been plotted in figures 1 to 3 with the growth time in weeks as abscissae and the weight of organisms as

TABLE 3

*Weights of tubercle bacilli which developed in five and eight weeks on synthetic media of various pH, with and without added iron, with and without citrate*

pH	PERIOD OF GROWTH	IRON ADDED, CITRATE ADDED	IRON ADDED, NO CITRATE ADDED	NO IRON ADDED, CITRATE ADDED
	<i>weeks</i>	<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>
6.0	5	24.8	24.2	22.1
6.5	5	25.6	30.9	22.4
7.0	5	29.9	31.5	27.4
7.4	5	39.7	32.4	28.2
8.0	5	29.8	27.0	23.5
6.0	8	29.8	30.6	28.8
6.5	8	38.6	45.3	29.5
7.0	8	56.7	51.1	35.1
7.4	8	94.0	45.0	33.2
8.0	8	47.2	36.5	29.5

ordinates. Since the weights of growth in corresponding cultures at pH 6.0 and 6.4 were very similar these results have been averaged and shown in one curve; likewise the figures for pH 7.0 and 7.4 have been averaged and the result shown in one curve.

The results presented in table 3 indicate that in this medium at pH 7.0 and 7.4, or the average of these, recorded as pH 7.2 in figure 2, in the absence of added iron the tubercle bacillus makes a relatively poor growth. Where iron and no citrate is added there is a slight increase in growth during the first five weeks and approximately twenty per cent increase in eight weeks. Where both iron and citrate are added there is considerable increase in

growth during the first five weeks over that which develops in the absence of iron, and an increase of more than a hundred per cent after eight weeks growth. At pH 8.0, as shown in figure 3, there is a similar though slightly less marked increase in growth from the addition of iron and both iron and citrate. In the more acid media there appears to be a slight advantage from the addition of the iron but very little advantage from the addition of the citrate. In the alkaline media the fact that the presence of citrate has relatively little influence during the first few weeks and great influence in the latter period of growth is probably the result of the fact, mentioned in the first section, that in these media in the absence of citrate while most of the iron is precipitated a trace remains (see table 1 and table 4); this trace is apparently sufficient for the initial growth of the tubercle bacilli. Later the favorable influence of the citrate in maintaining a larger amount of iron in solution becomes more apparent.

These results are in conformity with the conclusions reached by many previous observers that the presence of a small amount of iron promotes the growth of tubercle bacilli. It seems equally apparent that the utilization of the iron depends upon its maintenance in solution by the presence, in the neutral or alkaline culture fluid, of some such substance as citrate. We are not aware of any case where such stabilizing substances have been added to media of this type with this object in view but it is perhaps significant that some of the most successful synthetic media such as those of Long and Sibert (1926) have utilized ferric citrate.

#### IV. CHANGES IN THE IRON CONTENT OF MEDIA IN WHICH TUBERCLE BACILLI HAS GROWN

In table 4 the amount of iron in the original media used for the growth of tubercle bacilli, described in Section III, is compared with the amount present after the growth of the tubercle bacilli for five and eight weeks. The first column of this table indicates essentially as do the data in table 1, that the presence of citrate inhibits to a conspicuous degree the precipitation of iron. The actual amount of iron added to these media, as previously noted,

was, however, approximately half that used in the experiments summarized in table 1. Even in the presence of citrate the additions of more than approximately 0.10 gram of iron per liter results in precipitation during autoclaving.

The third and fourth columns in table 4, showing the amount of iron left in the media after the growth of tubercle bacilli, indicate comparatively little decrease in the iron content. It seems apparent, however, from these figures that the amount of decrease

TABLE 4

*Change in the iron content of the media with and without citrate during the period of growth of the tubercle bacillus*

INITIAL pH	IRON CONTENT			
	Initial after autoclaving	Five weeks growth of tubercle bacillus	Eight weeks growth of tubercle bacillus	Decrease eight weeks growth
With citrate				
	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>
6.0	0.082	0.050	0.036	0.046
6.5	0.097	0.076	0.080	0.017
7.0	0.101	0.084	0.072	0.029
7.4	0.109	0.082	0.085	0.018
8.0	0.096	0.090	0.078	
Without citrate				
	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>	<i>mgm. per cc.</i>
6.0	0.001	0.0006	0.0005	0.0005
6.5	0.0015	0.0009	0.001	0.0005
7.0	0.002	0.0019	0.0011	0.0009
7.4	0.002	0.0010	0.0009	0.001
8.0	0.001	0.0006	0.0002	0.0008

in iron during growth in the media containing both citrate and a relatively large concentration of soluble iron is as great or greater than the initial amount of soluble iron in the media to which no citrate was added.

The presence or absence of iron seemed to have no effect upon the production of acid products during the growth of the bacteria, the pH remaining relatively constant, probably as a result of the presence of the phosphates, but tending very slightly toward acidity in the more alkaline media.

# V. INFLUENCE OF IRON IN SYNTHETIC MEDIA WITH AND WITHOUT CITRATE ON THE GROWTH OF *B. LEPRÆ* AND *B. PHLEI*

For comparison with the growth of tubercle bacilli two other species of acid-fast organisms, *B. leprae* and *B. phlei* were grown on a similar series of synthetic media, with and without iron, and

TABLE 5

*Weights of B. leprae which have developed in six and in ten days in synthetic media of various pH with and without added iron and with the iron held in solution by the addition of sodium citrate*

pH	IRON ADDED, CITRATE ADDED		IRON ADDED, NO CITRATE		NO IRON, CITRATE ADDED	
	Six days	Ten days	Six days	Ten days	Six days	Ten days
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
6.0		25.8	11.3	22.2	9.5	10.4
6.4	15.2	27.8	15.4		11.1	13.4
7.0	16.0	26.8	7.2	24.3	13.4	11.7
7.4	16.1	24.8	13.4		10.5	20.1
8.0	13.1	30.3	26.2	29.5		22.1

TABLE 6

*Weights of B. phlei which have developed in six and in ten days in synthetic media of various pH with and without added iron and with the iron held in solution by the addition of sodium citrate*

pH	IRON ADDED, CITRATE ADDED		IRON ADDED, NO CITRATE		NO IRON, CITRATE ADDED	
	Six days	Ten days	Six days	Ten days	Six days	Ten days
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
6.0		26.4	9.6	17.1	15.5	9.5
6.4	27.5	25.4	11.5	26.7	17.3	15.4
7.0	28.3	37.0	17.2	32.9	14.1	27.1
7.4	22.3	37.5	25.4	30.7	23.4	18.0
8.0	36.2	42.0	30.1	42.8	19.2	36.2

with and without citrate. These were prepared in exactly the same manner as those used with the tubercle bacilli. After six days growth parts of the cultures were filtered, as previously described, washed, dried and weighed. After ten days growth the remaining cultures were similarly treated. The results of the growth of *B. leprae* (Clegg I, originally from the Lister Institute)

are shown in table 5 and results of the growth of *B. phlei* (also a culture from the Lister Institute) are shown in table 6.

Comparison of these figures indicates that with both *B. leprae* and *B. phlei* the least growth was in the solutions without added iron, that the addition of iron without citrate caused a slight increase in growth and that the addition of both iron and citrate caused a considerable increase in growth. The increase in growth associated with the presence of iron is, however, considerably less than in the case of the tubercle bacillus.

### CONCLUSIONS

Data have been presented to show that the addition of iron in the form of chloride or sulphate to a synthetic medium consisting of asparagin, glycerol,  $\text{Na}_2\text{HPO}_4$  and  $\text{NaCl}$  over the ordinary pH growth range of tubercle bacilli, results in almost, though not quite, complete precipitation of the iron. The addition of sodium citrate, probably through the formation of complex ions, inhibits the precipitation of iron over this pH range.

In a synthetic medium adjusted to pH 7.4, it has been shown that tubercle bacilli produce approximately 20 per cent more growth when iron and no citrate is added and slightly over 100 per cent more growth when both iron and citrate are added to the medium. In more acid and more alkaline media less conspicuous advantages result from the addition of iron. Similar results are obtained with the growth of *B. phlei* and *B. leprae* in these media.

### REFERENCES

- FROUIN, A., AND GUILLAUME, M. 1924 Compt. Rend. Soc. Biol., **90**, 878.  
 FROUIN, A., AND GUILLAUME, M. 1926 Bull. Soc. Chem. Biol., **8**, 1151.  
 HENLEY, R. R. 1925. Amer. Rev. Tuberc., **12**, 246.  
 HOPKINS, E. F., AND WANN, F. B. 1925 Jour. Gen. Physiol., **29**, 205.  
 LONG, E. R., AND SIEBERT, F. B. 1926 Amer. Rev. Tuberc., **13**, 393.  
 MARRIOTT, W. M., AND WOLF, C. G. 1905-1906 Jour. Biol. Chem., **1**, 452.  
 REED, H. S., AND HAAS, A. R. C. 1924 Bot. Gazette, **77**, 290.  
 SANTON, B. 1912 Compt. Rend. Acad. Sci., **155**, 860.





# VARIATIONS OF STREPTOCOCCI WITH A NOTE ON HEMOLYSIN PRODUCTION<sup>1</sup>

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Instances have been cited by Beitzke and Rosenthal (1906) Anthony (1909), Zoppritz (1909), Jaffe (1912), Rosenow (1914, A), and (1914, B), Hintze and Kuhne (1922), Pruska (1924), Morgenroth, Schnitzer and Berger (1925), Buerger and Ryttenberg (1907), Ludke and Polano (1909), Much (quoted by Hoessli, 1910), Jungman (1921), and others in which hemolytic streptococci (*S. hemolyticus*) have changed either spontaneously, or as a result of certain definite cultural procedures, into green producing streptococci (*S. viridans*). It is the object of this paper to show how error may arise from failure to recognize the true nature of a strain of streptococcus at the start of such investigations.

Strains of *Streptococcus viridans* are frequently found which, in rabbit-blood agar, pour plates, incubated *aerobically* for twenty-four to forty-eight hours, at 37°C., produce zones of hemolysis 2 to 4 mm. in width about the deep colonies. A photograph of such a blood agar culture is reproduced in figure 1. Such a strain of streptococci might be reported as *Streptococcus hemolyticus*. Certainly, if one regards as *S. hemolyticus* any strain showing a wide zone of hemolysis in blood agar pour plates (and it appears likely that this has been done in the past) then this strain would be regarded as of that type. Production of a zone of hemolysis of *any* width, however, is *not* the differential point.

Streptococci are of three main types as regards action in 5 per

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cent rabbit-blood, infusion-agar, pour plates incubated aerobically at 37°C. for twenty-four to forty-eight hours. Two of these are hemolytic. The third (Gamma type—Brown, 1919) produces no change whatever and will not be discussed further in

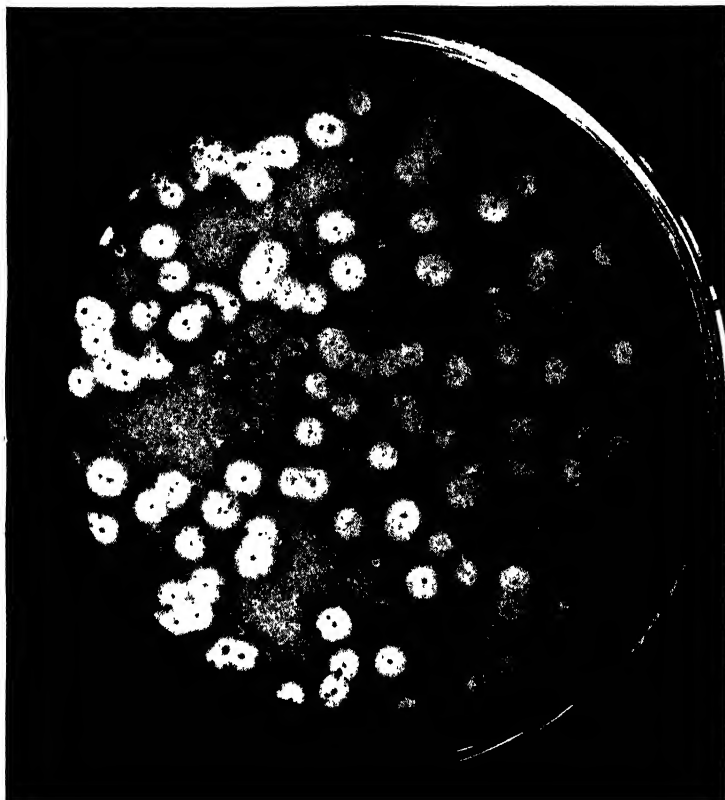


FIG. 1. BLOOD AGAR PLATE CONTAINING A MIXED CULTURE OF BETA TYPE AND ALPHA TYPE STREPTOCOCCI OF THE EXTREMELY HEMOLYTIC SORT MENTIONED IN THE TEXT

The alpha type colony with the ring around it is the one shown in figure 2

this paper. Of the two hemolytic types, one produces about its colonies a zone of hemolysis devoid of visible blood corpuscles. This is the beta type of Smith and Brown (1915); Brown (1919). (Most strains of the so-called *S. hemolyticus* reported in the litera-

ture are probably of this type but it is also probable that some of the more hemolytic alpha type strains have been mistaken for the beta type.) The other type also produces a zone of more or less hemolysis about its colonies. The production of the hemolytic zone is *preceded* by the production of a zone, close around the colony, of methemoglobinized corpuscles. This is the alpha type of Smith and Brown (1915); Brown (1919). *S. viridans* belongs to this type. There are also strains producing no grossly visible greenish color which belong to the alpha type. The corpuscles in this inner zone, once methemoglobinized, are not subject to hemolysis. Consequently, when hemolysin is produced later in the life of the colony, the blood cells remain around the colony as a more or less greenish or brownish zone. The clear zone appears only when, and if, the hemolysin is produced in sufficient amount to diffuse beyond the zone of discolored corpuscles. For brevity these zones are here referred to as the "green" and "clear" zones respectively. The relative and absolute widths of the green and clear zones are prone to vary, sometimes within wide limits, as described in this paper. One zone may at times be so exaggerated as completely to mask the other. The literature contains a large number of reports of such variations.

The hemolytic character of *S. viridans* is easily demonstrated by the anaerobic cultivation of the pour-plate cultures (Brown, 1919). Here the oxidation processes which are partly responsible (Hagan, 1925, and Brown, 1919) for the green zone are inhibited and the colonies so cultivated are indistinguishable from those of hemolytic (beta type) streptococci.

In some cases, hemolysin production by alpha type streptococci occurs so early and so extensively as to make the green zone very narrow both absolutely and relatively. The green zone in such cases escapes notice unless the low power of the microscope be focussed upon the deep colony, when the true nature of the growth is made clearly visible. As a further check, recourse may be had to the effect of 1 cc. of a young, (eight to twelve-hour) *well grown*, 20 per cent horse serum, broth culture (de Kruif and Ireland, 1920) upon 1 cc. of a 5 per cent suspension of washed rabbit erythrocytes. The mixture is held in the water bath at

37°C. for one or two hours. Under such conditions, cultures of alpha type streptococci produce no trace of hemolysis. Methemoglobin production, with the development of brownish, greenish or purplish tints may be pronounced. By the routine use of the microscope, supplemented, in cases of doubt, by the test tube hemolysin test just described, one avoids error in the designation of streptococci as alpha or beta types.



FIG. 2. APPEARANCE OF THE ALPHA TYPE COLONY MARKED WITH A RING IN FIGURE 1, WHEN VIEWED WITH THE LOW POWER OF THE MICROSCOPE

The zone of discolored corpuscles close around the colony is clearly visible

The tube hemolysin test must not be used as a substitute for microscopic examination of deep colonies in blood agar, because there are streptococci which, with the microscope, are clearly seen to be of the beta type, yet yield no test tube hemolysis.

#### EXPERIMENTAL

Fifty strains of streptococci producing wide zones of hemolysis, thirteen of which were considered, by gross inspection of the plate, as *S. hemolyticus* (beta type), and thirty-seven of which produced

no grossly visible methemoglobinized zone, were subjected to the tests described above. Figure 1 shows the gross appearance of one of the pour plates and figure 2 shows the microscopic appearance of one of the colonies, with the zone of methemoglobinized cells clearly visible. The test-tube hemolysin test was absolutely negative in every case. The possibility of error through failure to use microscopic examination of colonies and the tube hemolysin test in the study of streptococci is quite evident.

It seems desirable to point out in this connection, particularly in view of a statement by Holman (1916) and the proposed classification of pseudo-hemolytic streptococci by Cumming (1927), that colonies of either alpha or beta type streptococci may show little or no hemolysis or green production when growing *on the surface* of blood agar plates. It is extremely difficult in some cases (not all) to be certain of the nature of a surface colony. Holman's reason for grouping the alpha and gamma (indifferent) streptococci together is based on surface streak methods. Cumming's proposed classification is similarly based on this technique. In the latter study the tube hemolysis tests were made using 0.2 cc. of a twenty-four-hour culture plus 1 cc. of 3 per cent corpuscle suspension. Many workers have shown that hemolysin is extremely labile and reaches a maximum concentration early in the life of the culture, often disappearing after fourteen hours. Tests made after fourteen hours, therefore, may often give falsely negative results. It has also been observed by many workers that true, beta type hemolytic streptococci vary greatly in their hemolytic powers. The use of 0.2 cc. of a broth culture might therefore exclude from consideration many weakly hemolytic, but genuine, beta type streptococci. According to the proposed classification, streptococci failing in these tube hemolysin tests but giving wide zones of hemolysis on the surface of blood agar plates were classed as pseudo-hemolytic streptococci. It appears, then that such a group would include (a) slightly hemolytic beta type streptococci, (b) strongly hemolytic alpha type streptococci, and (c) strongly hemolytic pneumococci.<sup>3</sup> This grouping would offer no advantages over the present systems.

<sup>3</sup> Pneumococci are exactly like alpha type streptococci in respect to blood agar and test-tube hemolysin tests.

## VARIATIONS

The fifty strains referred to above were first kept for varying periods upon blood agar slants with monthly renewals. In May 1927 they were dried on pieces of sterile filter paper in vacuum jars (Brown, 1925, 1926) and held in the refrigerator until Febru-

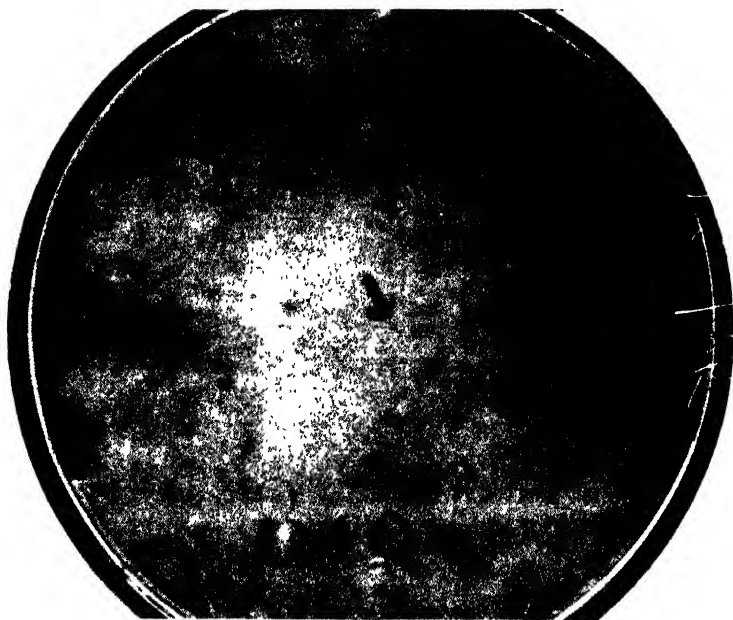


FIG. 3. BLOOD AGAR PLATE CULTURE OF THE SAME ALPHA TYPE STREPTOCOCCUS AS THAT SHOWN IN FIGURE 1

This culture was made nearly two years later, after the organism had lost its exaggerated hemolytic power. The arrow almost touches a colony. Due to the very small zones, a good picture could not be made. The contrast between this plate and figure 1, however, is marked, and the plate is included as a matter of record.

ary, 1928. At this time all were plated out in the same manner as previously described. All were found to have lost their exaggerated hemolytic properties and to have reverted to the ordinary alpha type usually recognized as such without difficulty. Figure 3 shows the appearance under these conditions, of a blood agar plate culture of the same streptococcus as that shown in figure 1.

Without microscopic examination and tube hemolysin test of the streptococcus under its original conditions, one might have concluded that a beta type streptococcus had mutated to an alpha type streptococcus. It is suggested, in the light of these illustrations, that many of the cases reported as mutations of streptococci are due to faulty observation and failure to recognize the type of streptococcus in the first place coupled with a diminution (not loss) of hemolytic power in a genuine alpha type streptococcus.

#### SUMMARY AND CONCLUSIONS

1. For the study and proper classification of streptococci in blood agar the following are essential:

a. Differentiation of types based on low power microscopic observation of deep and not of surface colonies in blood agar plates.

b. Use of tube hemolysin tests with properly prepared cultures to *supplement* plate observations.

2. Genuine, alpha type streptococci have frequently been encountered which possess very marked hemolytic powers, producing hemolytic zones 2 to 4 mm. wide and appearing, to the naked eye, to be beta type (hemolyticus) streptococci. These strains never caused hemolysis when their broth cultures were mixed with red cell suspensions. When the deep colonies of such streptococci in blood agar pour plates were viewed with the low power microscope, they were seen to have about them, *inside* the hemolytic zone, the zone of methemoglobinized cells common to all alpha type streptococci.

3. A number of such streptococci have been found to lose a large part (not all) of their hemolytic powers after being kept for some time under various artificial conditions and to appear like the ordinary and easily recognized alpha type of streptococcus. The diminution of hemolytic properties may be due to a change in the organisms or to some obscure variation in technique.

4. It is suggested that some of the reported mutations in which a beta type streptococcus has been said to change into an alpha type streptococcus may be due to failure to recognize the true character of the organism in the first place, accompanied by a diminution of the original hemolytic properties.



5. It is possible that many of the anomalous results of many sorts reported by investigators of streptococci may be due to confusion in identification of alpha and beta types.

## REFERENCES

- ANTHONY, B. VAN H. 1909 Some characteristics of the streptococci found in scarlet fever. *Jour. Inf. Dis.*, **6**, 332.
- BEITZKE, H., AND ROSENTHAL, O. 1906 Zur Unterscheidung der Streptokokken mittels Blutnährboden. *Ark. pathol. Inst. Berlin*, 349.
- BROWN, J. H. 1919 Blood agar for the study of streptococci. *Monographs of The Rockefeller Institute*, Number 9.
- BROWN, J. H. 1925 The preservation of bacteria in vacuo. *Abs. Bact.*, **9**, 8.
- BROWN, J. H. 1926 Vacuum tubes for the storage and shipment of bacteria. *Science*, **64**, 429.
- CUMMING, W. M. 1927 On the pseudohemolytic streptococci isolated from the sputum in pulmonary tuberculosis. *Jour. Pathol. and Bact.*, **30**, 279.
- DEKRUIF, P. H., AND IRELAND, P. M. 1920 Streptolysin. *Jour. Inf. Dis.*, **26**, 285.
- HAGAN, W. A. 1925 The green coloration produced by certain streptococci on blood-agar plates. *Jour. Inf. Dis.*, **37**, 1.
- HINTZE, K., AND KUHN, R. 1922 Zur Frage Umwandlung haemolytischer Streptokokken in die Grün-Wachsende Form. *Zentralbl. f. Bakt.*, 1 abt., Orig., **88**, 352.
- HOLMAN, W. L. 1916 The classification of streptococci. *Jour. Med. Res.*, **34**, 377.
- JAFFE, R. 1912 Beobachtungen bei blutlosenden und bei Gramnegativen Streptokokken. *Arch. f. Hyg*, **76**, 137.
- JOPPRITZ, B. 1909 Ueber Streptokokken versuche. *Med. Klin*, **5**, 1112.
- JUNGMAN, P. 1921 Zur Klinik und Pathogenese der Streptokokken. *Deut. Med. Woch.*, **47**, 496.
- LUDKE AND POLANO 1909 Ueber Hemolyse der Streptokokken. *Münch. Med. Woch.*, **56**, 7.
- MORGENROTH, J., SCHNITZER, R., AND BERGER, E. 1925 Ueber die Einheit der Streptokokken und Pneumokokken. 1 Mitteilung. *Ztschr. f. Immunitätsforsch. u exp. Therap. Teil 1*, Orig., **43**, 169.
- MUCH, H. (Quoted by Hoessli). 1910 Cited from Brown, J. H., 1919. loc. cit.
- PRUSKA 1924 Die Umwandlung des haemolysierenden in einem Nicht-haemolysierenden Streptokokkus. *Centralbl. f. Bakt.*, 1, abt., Orig., Ref. **77**, 208.
- ROSENOW, E. C. 1914a Transmutations within the streptococcus-pneumococcus group. *Jour. Inf. Dis*, **14**, 1.
- ROSENOW, E. C. 1914b The etiology of acute rheumatism, articular and muscular. *Jour. Inf. Dis.*, **14**, 61.
- SMITH, T., AND BROWN, J. H. 1915 A study of streptococci isolated from certain presumably milk-bone epidemics of tonsillitis occurring in Massachusetts in 1913 and 1914. *Jour. Med. Res.*, **31**, 455.

## STREPTOCOCCUS STUDIES

### I. STREPTOCOCCUS VIRIDANS DERIVED FROM SINGLE CELL STRAINS OF STREPTOCOCCUS HEMOLYTICUS

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Changes in those supposedly basic characteristics on which bacteria have been conveniently and—so far as pathogenic bacteriology is concerned—quite appropriately classified are being observed with a frequency which has already begun to modify our conceptions of the limitations of variation in morphology and in cultural characteristics within what have been accepted as fixed bacterial species.

Variations within the genus *Streptococcus* which would not only change a given organism from one species to another, but from one group to another have, in the light of recent advances in our knowledge of scarlet fever, erysipelas and other streptococcus diseases, not only an interest for purposes of classification, but have acquired a considerable etiological and pathological significance.

Our present communication—the first of a series of streptococcus studies—is based chiefly upon the changes which may occur in cultures of hemolytic streptococci.

Valentine and Krumwiede in 1922 observed the sudden appearance of green colonies in a culture of a hemolytic streptococcus which had never shown such colonies when plated out at frequent intervals during more than a year of observation. Both varieties of colony bred true to type in subculture. They were alike in fermentation reactions and were serologically identical. The beta strains killed mice regularly in 0.5 cc. amounts and occasionally in 0.25 cc. doses, whereas the alpha strains killed only irregularly in 1.0 cc. and 1.5 cc. amounts of eighteen-hour broth cultures. The authors were unable to obtain viridans

colonies from frequent plating of twenty other strains of hemolytic streptococci.

Morgenroth, Schnitzer and Berger (1925) report changing *Streptococcus hemolyticus* into *Streptococcus viridans* by growth in broth containing rivanol; but Reimann (1927) was unable to confirm these results.

Lehmann (1926) obtained green-producing colonies from three out of eighty-seven strains of hemolytic streptococci which had been cultivated in milk media.

Kurokawa (1927) reported obtaining green colonies from two out of five strains of hemolytic streptococci by exposure to rivanol and observed a gradual change from hemolysis to green production accompanied by a progressive loss of virulence, and also a similar return of hemolytic capacity and virulence when these strains were grown in the "usual media" or passed through a series of twelve or thirteen mice.

In plating out four strains of hemolytic streptococci from scarlet fever, it was observed that a varying number of colonies of the alpha type appeared on certain of the plates from two of these strains, while their presence was never seen in plates of the other two strains.

It seemed worth while to ascertain whether single cell strains of these cultures would give rise to colonies of the alpha type. Four single cell cultures were therefore made from each of the two strains which had shown alpha colonies. Each single cell strain was carried through daily subcultures in two varieties of broth containing, respectively, 20 per cent normal horse serum and 20 per cent anti-streptococcus serum, on blood agar slants at 37°C. and on blood agar slants at 40°C.

Plates from the fourteenth subculture of one strain in normal horse serum broth and from the twenty-first subculture of another strain on blood agar incubated at 40°C. showed a few colonies of the alpha type. Three colonies were picked from each of these plates and single cell strains obtained from cultures of each colony. These alpha type strains resemble each other in their gross appearance on blood agar plates in that the colonies are smaller than the colonies of the beta type culture from which they were derived, and instead of showing a wide zone of clear hemolysis they are surrounded by a narrow ring of green. They differ,

however, from each other in certain respects. Five of them show "smooth" colonies on blood agar, while the sixth is "rough." Three grow with uniform turbidity in broth, and three give a flocculent growth. Four strains ferment glucose, lactose, sucrose and salicine, as does the beta strain, one ferments also raffinose and one fails to ferment salicin.

The single cell beta type culture, from which these alpha strains were derived, kills mice regularly in 0.1 cc. amounts and occasionally with 0.05 cc. Two cubic centimeters of a twenty-four hour hormone broth culture of five of the alpha strains fail to kill mice. The sixth strain kills uniformly with 2.0 cc. and kills about half the mice receiving 1.0 cc.

TABLE 1

*Scarlet fever strain B, showing occasional green colonies in single cell strains*  
Daily sub-cultures in broth and on blood agar as described

SCB1*	SCB2	SCB3	SCB4
14 sub-cultures in normal horse serum broth	21 sub-cultures on blood agar at 40°C.	30 sub-cultures in normal horse serum broth	30 sub-cultures on blood agar at 37°C.
Alpha colonies in 14th generation <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">             †ASC1              /           </div> <div style="text-align: center;">             ASC2                          </div> <div style="text-align: center;">             \             ASC3           </div> </div>	Alpha colonies in 21st generation <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">             ASC4              /           </div> <div style="text-align: center;">             ASC5                          </div> <div style="text-align: center;">             \             ASC6           </div> </div>	No alpha colonies	No alpha colonies

\* SCB = single cell beta.

† ASC = single cell alpha.

The single cell beta strains grown in anti-streptococcus serum and on blood agar at 37°C. showed no alpha colonies during thirty daily subcultures, nor did alpha colonies appear again in the cultures in normal horse serum broth nor on blood agar at 40°C. (See table 1.)

A further instance in which a hemolytic streptococcus gave rise to colonies of the viridans type, but one which unfortunately did not involve single cell isolation, occurred under the following conditions: Four strains of scarlet fever streptococci were incubated on blood agar slants for twenty-four hours and plated out.

These plates showed hemolytic colonies only. The slants were now sealed and kept in the refrigerator for seventy-five days, and then replated. Three of these plates showed hemolytic colonies only; but the plate of the fourth strain showed about 90 per cent of colonies of the alpha type.

Subcultures of the beta type colonies produce typical hemolytic colonies on blood agar and flocculent growth in broth, whereas subcultures of the alpha colonies produce "smooth" green colonies on blood agar and grow with uniform turbidity in broth. The beta strains ferment glucose, lactose, sucrose and salicin, while the alpha strains ferment also raffinose. The difference of the two strains in virulence for mice is slight. One cubic centimeter of the beta strain kills mice regularly, whereas the same dose of the alpha strain kills only occasionally.

Work is now in progress to determine whether, under suitable conditions, single cell strains of the alpha type may show a reversion to the beta type of growth and an increase of virulence. The toxin-producing power of these green producing derivatives of scarlet fever streptococci is also being studied.

#### DISCUSSION

A considerable number of reports have now appeared in the literature of instances in which colonies of the viridans type of streptococcus have appeared in certain cultures which ordinarily show only the hemolytic type of growth, and other cases in which, by special methods of cultivation, the hemolytic power of whole cultures has been gradually lost and the ability to produce methemoglobin has been acquired.

It has now been shown that single cells of hemolytic streptococci may give rise to daughter strains with the characteristics of *Streptococcus viridans* and that these variant strains remain true to type under ordinary methods of cultivation. The ability to give rise to green producing strains would seem to be possessed by certain streptococci only. The variant forms appear only occasionally and under a number of different cultural conditions.

The streptococci have usually been classified by their fermentation reactions, by their effect on blood agar or by a combination

of the two. But if a single cell of one of these forms can give off daughter strains of another type, these classifications, while still convenient for general use, naturally lose their biological significance.

It is unnecessary to regard these variations in the streptococci as in the nature of mutations; but rather as indications that our present conceptions of the limits of certain at least of the bacterial species are too narrow.

#### SUMMARY AND CONCLUSIONS

1. Single cell strains of hemolytic streptococci may under certain conditions of cultivation give rise to daughter strains of the alpha type.

2. These alpha variants resemble in every respect *Streptococcus viridans*.

3. The change from the beta to the alpha type occurs only with certain strains. The change is characterized by the occasional appearance of alpha type colonies, and there is not as a rule a complete change of the whole culture to the alpha type similar to the change from S to R with pneumococci.

4. The alpha variants remain true to type with no tendency to revert under the usual conditions of cultivation.

5. The loss of hemolytic power is accompanied by a slight loss of virulence.

6. The alpha derivatives usually but not always give the same fermentation reactions as the beta strains from which they are derived.

#### REFERENCES

- KUROKAWA, AYAHIRO. 1927 Tokoku Jour. Exp. Med., **9**, 355.  
LEHMANN, W. 1926 Deutsch. Arch. Klin. Med., **150**, 127.  
MORGENROTH, SCHNITZE AND BERGER 1925 Ztsch. f. Immunitätsforsch., **43**, 209.  
REIMANN, HOBART A. 1927 Jour. Exp. Med., **45**, 1.  
VALENTINE, E., AND KRUMWIEDE, C. 1922 Jour. Exp. Med., **36**, 157.



# HEMOLYTIC REACTIONS OF A PATHOGENIC BOVINE STRAIN OF *B. COLI*

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The hemolytic reactions reported in this paper were carried out in an attempt to determine something about the nature of the hemolytic agent of a *B. coli* strain. This strain belonged to a group of pathogenic *B. coli* cultures from bovine sources which have been described by T. Smith and his associates (1927). Only one among twelve of these strains gave a hemolytic reaction and this strain (1192) occurred in two forms, the original *a* and mutant *b* types. The *a* type culture was originally isolated from the ileum of a calf suffering from scours and it is characterized as a non-sucrose-fermenting strain, non-motile, encapsulated, and forming thick, opaque, viscid colonies. From these colonies there developed a thin, partly translucent mutant growth (*b*) containing organisms without a capsule which are less virulent and more easily agglutinated than the *a* type. Two other *B. coli* strains isolated more recently from the spleen and ileum of the same calf (1369) were also tested for hemolysis with the result that the spleen culture was found to be hemolytic and the ileum culture non-hemolytic. Strain 1369 (spleen) as far as tested, corresponds with 1192 in its hemolytic properties. For a period of a year both the *a* and *b* types of 1192 were alike in their hemolytic reactions and then a non-hemolytic *b* form appeared. Since both *a* and *b* (hemolytic) forms showed essentially the same hemolytic properties, results with only one of the strains have been recorded, although most tests were carried out with both cultures. The new variant *b* (non-hemolytic) will be referred to later in the paper.



In the literature, Kolle and Wassermann (1913) list a number of reports by various authors recording the presence of hemolytic strains of *B. coli*. More recently Dudgeon, Wordley and Bawtree (1921-1922) described hemolytic *B. coli* strains isolated from urinary tract infections and from various samples of feces. They tested the hemolytic reactions in peptone water cultures with human blood corpuscles.

All the experiments reported in this paper were made with the *B. coli* strain 1192. Cultures in both fluid and solid media were used but the majority of the tests were carried out with fluid cultures.

The first experiment with Strain 1192 demonstrated the existence of its hemolytic property and its ability to attack corpuscles from various animals. Horse, sheep, cow, rabbit, guinea pig, and human corpuscles were used. The test was made by mixing 0.5 cc. of a twenty-four-hour bouillon culture with 0.5 cc. salt solution. The corpuscles were washed three times and suspended in salt solution to the original volume of the blood. One drop of this suspension was added to the 1 cc. of culture dilution and the mixture incubated at 37°C. for two hours and refrigerated overnight. The results showed strong or complete hemolysis of all corpuscles.

In the following experiments horse corpuscles were used. First a group of experiments was made to test the hemolytic activity of cultures after the bacilli had been killed or removed. The results showed that the disappearance of the hemolytic activity was coincident with the destruction or removal of the living bacteria. This was shown first when twenty-four-hour bouillon cultures were heated thirty minutes at temperatures from 45° to 100°C. Exposure at 60°C. and above prevented hemolysis and killed the bacteria. In general the same was true of four-hour cultures. However, when the test was made immediately after the heating a trace of hemolysis occurred in some tubes, but if repeated later no hemolysis was visible. In the second experiment chloroform or ether was used to kill the cultures since these substances are known to kill bacteria but leave present bacterial products such as enzymes. Hemolytic tests with these cul-

tures were negative. Finally the bacteria were removed by passing the cultures through Berkefeld filters. Filtrates from four-, twenty-four- and forty-eight-hour and seven-day cultures were all non-hemolytic. An attempt was made to remove the bacteria by centrifuging. Cultures centrifuged one-half hour and four hours were perfectly clear, but this clear supernatant fluid caused hemolysis similar to that produced by whole cultures. Tests showed that a number of bacteria were still present in the clear centrifugate and that these multiplied during the test period. Different samples of the centrifugate may show varying degrees of hemolysis probably depending on the number of organisms in the fluid. This group of experiments definitely associates the hemolytic agent with the living bacilli.

The next group of experiments includes tests on the strength of the hemolytic agent in different cultures. First a twenty-four-hour agar and bouillon culture were compared by suspending the agar growth in salt solution to the density of the bouillon culture. This comparison showed that as a rule the agar growth produced a lower degree of hemolysis than the bouillon culture unless the agar suspension was diluted 1:1 with bouillon. This reaction may be explained by the fact that the bouillon stimulated a more rapid growth of bacilli during the test period than occurred in the salt solution suspension alone. In the second place bouillon cultures of different ages were tested to see at what time in the growth of a culture the strongest hemolytic action occurred. Cultures from two to forty-eight hours old were used and the strength of the hemolytic agent measured by the length of time required for complete hemolysis to take place. The results indicated that the hemolytic activity was greatest in a four-hour culture.

Next an experiment was made to see if the hemolytic agent was produced when the cultures were grown under anaerobic conditions. The tests were made in open tubes and also in tubes sealed with vaseline. The results of several experiments showed that with proper dilution a strong hemolytic activity could be demonstrated in these cultures. Thus the hemolytic agent is produced during anaerobic growth, and the fact that the hemoly-

tic titer was sometimes lower in the 1:1 than in the 1:10 dilution may be explained by the formation of an inhibiting substance which interferes with the hemolytic activity of the culture unless it is sufficiently diluted.

There is also a difference in the hemolytic action of aerobic cultures depending on dilution as the next group of experiments demonstrates. Up to this time the hemolytic tests had always

TABLE 1

*Comparison of hemolytic actions of bouillon cultures, undiluted, diluted 1:1 with salt solution, and diluted 1:1 with bouillon*

BOUILLON CULTURE 1192 b	DILUTED 1:1 WITH SALT SOLUTION			UNDILUTED			DILUTED 1:1 WITH BOUILLON		
	37°C.		Refrig- erated over- night	37°C.		Refrig- erated over- night	37°C.		Refrig- erated over- night
	1 hour	2 hours		1 hour	2 hours		1 hour	2 hours	
hours									
2	++	++++	C	-	-	+++	-	-	++
4	C	C	C	-	-	++	-	-	++
6	++++	C	C	-	-	++			
8		++++	C	-	-	++			
16	++	C	C	-	++	C			
18	+	+++	++++	-	-	+++			
20	++	++++	C	-	-	+			
24	++	C	C	-	±	++++	-	-	++
30	+	++	++++	-	-	++			
48	±	++++	C	-	-	+			
54	+	+++	++++	-	-	+	-	-	+

C = complete hemolysis; +++++, strong hemolysis but a few corpuscles still unhemolyzed; +++, ++, +, decreasing degrees of hemolysis with increasing amounts of corpuscles left in tube; ±, a just visible trace of hemolysis; -, no visible hemolysis.

been made by adding 0.5 cc. salt solution to 0.5 cc. of bouillon culture. When undiluted culture was used in the hemolytic test the low titer obtained was unexpected. Therefore a series of cultures from two to fifty-four hours old were compared for their hemolytic reactions in undiluted portions and portions diluted with equal volumes of salt solution. The results are found in table 1, columns 1 and 2.

In practically every case the undiluted culture gave a slower

reaction and lower final titer than the culture diluted with equal volumes of salt solution. These different reactions apparently are not due to changes in the H-ion concentration caused by addition of salt solution, since this changed the pH only slightly and, as shown later, cultures within a pH range of 6.0 to 8.0 produce about the same degree of hemolysis. Also the explanation that an inhibitory substance is formed in the culture is not wholly satisfactory, as demonstrated in the next experiment in which bouillon was used as the diluent.

A comparison between cultures diluted with salt solution and with fresh bouillon (table 1) showed that the cultures diluted with bouillon behaved like undiluted cultures. This suggested the presence of an inhibiting substance in bouillon rather than the formation of such a substance by growth of the culture. This was upheld in other experiments, namely, (1) one in which dilutions with bouillon were made throughout a longer series, (2) one comparing results with different lots of bouillon, and (3) experiments with filtered bouillon. When tests such as those recorded in table 1 were made with cultures grown in different lots of bouillon in some instances the undiluted culture showed a delayed reaction only at the first hour reading.

In an attempt to see if filtering would remove any substance from the bouillon and so change its reaction toward hemolysis, a bouillon which showed this inhibiting effect was passed through a Berkefeld filter. The filtered bouillon was used in two experiments as follows. First, a comparison was made of the hemolytic action of a bouillon culture diluted 1:1 with whole bouillon, filtered bouillon, and salt solution. The results showed an agreement in the hemolytic titer of tubes containing dilutions with salt solution and filtered bouillon. In a second experiment the culture was grown in whole and filtered bouillon and the hemolytic titer of the two cultures compared. The filtered bouillon culture gave a strong reaction when tested without diluting and its titer was increased slightly or not at all by dilution with salt solution, both reactions being in contrast to those of the whole bouillon culture whose hemolytic titer was low in the undiluted portions and greatly increased when diluted with equal parts of

salt solution. These results indicate that filtering bouillon through a Berkefeld filter removes some substance which has an inhibiting effect on the hemolytic activity of culture 1192.

The inhibiting factor of bouillon may be due to the added peptone or to some substance in the veal infusion broth or to some combination of these substances. In an attempt to determine which of these portions contained the inhibiting substance experiments were made with (1) veal infusion broth containing no added peptone, (2) veal infusion broth plus different amounts of peptone from 1 to 5 per cent, and (3) peptone water solutions in concentrations from 1 to 5 per cent. The results showed in the peptone-free veal infusion broth a positive hemolytic reaction equally strong whether the culture was undiluted or diluted 1:1 with salt solution. Also 1 per cent peptone bouillon allowed hemolysis as usual but almost complete inhibition of hemolysis occurred in the 2 per cent peptone bouillon although growth developed in this tube. Peptone water cultures containing 1 to 4 per cent peptone produced hemolysis while 5 per cent peptone water inhibited hemolysis. These results indicate, first, that peptone-free veal infusion broth contains no directly inhibiting factor; second, that peptone in certain concentrations possesses an inhibiting power; and third, that since 2 per cent peptone in bouillon interferes with hemolysis whereas 5 per cent is necessary in the water solutions, the inhibiting effect of bouillon is probably due to the combination of some factor in the veal infusion with the peptone. This is also suggested by the fact that filtration of 5 per cent peptone water does not seem to remove any inhibiting factor from the solution, whereas filtration of the bouillon does remove some inhibiting substance. Therefore the inhibiting substance in bouillon differs from that in peptone, and if it is due to the peptone it is also associated with some bouillon factor that makes it removable by filtering.

In connection with the filtration experiments, the effect of culture filtrates on the hemolytic action of fresh cultures was tested. Cultures diluted with these filtrates gave a higher titer than undiluted cultures or those diluted with whole bouillon, but not quite as strong a reaction as cultures diluted with filtered bouillon or salt solution.

TABLE 2  
*Effect of pH on hemolysis by B. coli 1192*

I. CULTURE GROWN IN ONE LOT OF BOUILLON AND ADJUSTED TO A SERIES OF pH VALUES AFTER GROWTH									
pH	Adjusted after 4 hours growth			Adjusted after 24 hours growth			Tested after 4 hours growth		
	Hemolysis			Hemolysis			Hemolysis		
	1 hour, 37°C.	Refrigerated overnight	37°C.	1 hour	2 hours	Refrigerated overnight	Growth	37°C.	Refrigerated overnight
5.0	+	C*	—	—	—	++*	None	—	C*
5.2	+	C*	—	—	—	++*	None	—	+
5.4	+++	C	—	—	—	+	Trace	—	—
5.6	C	C	—	—	—	+	Trace	—	—
5.8	C	C	—	—	—	++	Slight	—	+
6.0	C	C	—	—	—	+++	Definite	—	++
6.2	+++	C	—	—	—	C	Definite	—	+
6.4-6.5	+++	+	—	—	—	C	Definite	—	C
6.8	+++	C	—	—	—	C	Definite	—	C
7.0-7.1	C	C	—	—	—	C	Definite	—	C
7.2-7.4	+++	C	—	—	—	C	Definite	—	C
7.6-7.8	+++	C	—	—	—	C	Definite	—	C
8.0	C	C	—	—	—	C	Definite	—	C
8.2	+++	C	—	—	—	C	Definite	—	C
8.4	+++	C	—	—	—	C	Definite	—	C
8.6	+++	C	—	—	—	C	Definite	—	C
8.8	+++	C	—	—	—	C	Definite	—	C

C = complete hemolysis; ++++, strong hemolysis but a few corpuscles still unhemolyzed; ++, decreasing degrees of hemolysis with increasing amounts of corpuscles left in tube; ±, a just visible trace of hemolysis; —, no visible hemolysis.

\* Brownish, acid.

Two types of experiments were made on the effect of the H-ion concentration on the hemolytic activity of the special strain. First, the culture was grown in one lot of standard bouillon, the original pH of which was 7.1. After growth the culture was divided into portions and adjusted to a series of pH values from 5.0 to 8.4 by adding HCl or NaOH. These portions were then tested for their hemolytic reactions. In the second experiment tubes of different original pH values were inoculated and tested for hemolysis after growth. Fermented bouillon was used in the second experiment since in standard bouillon a considerable change of pH occurred with growth. The results of these two experiments are recorded in table 2. Except where hemolysis was directly due to the acid, a low or negative hemolytic titer usually existed in tubes below pH 6.0 which was apparently due to decreased growth of the culture in these tubes. The hemolytic agent seems to be interfered with more readily by acid than by alkali. These experiments again indicate that the hemolytic agent is a product of the living bacteria and the pH affects the hemolytic agent whenever it interferes with the growth of the culture.

In order to determine whether or not the hemolytic agent was active at refrigerator temperature both whole cultures and centrifugates were mixed in the usual way with salt solution and corpuscles and refrigerated immediately. After two hours no hemolysis was visible but after twenty hours a slight hemolysis occurred. Colony counts showed that the number of bacteria in the refrigerated suspensions was about the same or less than the number at the beginning of the test, indicating that the rate of multiplication in these tubes had been low. Apparently the hemolytic agent is able to act at refrigerator temperature and the slight reaction obtained in these tubes is probably due to a decreased production of the hemolytic agent rather than to its inability to act at the low temperature.

In the following experiment an attempt was made to find out something about the hemolytic agent by comparing media in which the culture did and did not produce hemolysis. Previous experiments showed that the hemolytic agent was formed by cul-

tures grown in peptone water alone and also in peptone-free veal infusion broth. When salt solution plus 1 per cent glucose was tried as a medium, inoculation with a loopful of a bouillon culture caused a definite clouding to develop but no hemolysis. These results indicate that the hemolytic agent can be produced from peptone alone and also from certain media containing no peptone, such as a veal infusion broth, but that salt solution and glucose do not supply the proper material for the formation of the hemolytic agent.

Finally it was thought that the nature of the hemolytic agent might be revealed to a certain extent by means of the kind of substances which inhibited its reaction. So far, certain lots of standard bouillon, 5 per cent peptone water, and 2 per cent peptone bouillon have been noted to interfere with the hemolytic activity of the culture. Other inhibiting agents that have been found are certain normal sera, especially horse, cow, and human sera, and certain concentrations of cholesterol. Also specific immune serum shows an increase or original rise in inhibiting power as a result of immunization. This result brings out another point concerning the nature of the hemolytic agent, namely, that it may possess antigenic properties. These serum experiments are recorded in detail in a second paper dealing with the inhibiting effect of sera on hemolysis. All these inhibiting substances prevent hemolysis only in definite concentrations, and if sufficiently diluted do not interfere with the hemolytic activity of the culture.

In regard to experiments on solid media, when cultures were plated on plain agar plus washed corpuscles large, clear, hemolytic zones 2 to 4 mm. in diameter developed around the deep colonies and clear zones extended 1 to 2 mm. beyond the edges of the surface colonies. In contrast to this, if whole defibrinated horse blood containing the serum was used instead of washed corpuscles, only a ring of hemolysis was visible around the deep colonies and only the corpuscles directly below the surface colonies were hemolyzed. Thus on agar cultures as well as bouillon the hemolytic activity and the inhibiting action of serum on hemolysis can be demonstrated. The inhibiting action of the serum did not appear as complete in the agar as in the bouillon culture, but the small



hemolytic zones on the serum plates may be explained by assuming that the serum in the vicinity of the colony gradually becomes used up in combining with the hemolytic agent. As the colony keeps on growing the hemolytic agent continues to be formed, so eventually some is free to act on the red cells; whereas in the bouillon culture the serum comes in contact more readily with the hemolytic agent and so can exert its inhibiting power more completely.

In connection with the agar plate experiments an acid hemolysis produced by the culture was also demonstrated. If glucose agar was used, in addition to the distinct hemolytic zones there was a brownish discoloration and a more or less general clearing of the plate depending upon how thickly it was inoculated. The culture ferments the glucose with acid production and the resulting acid hemolyzes the blood. This acid hemolysis is of a more spreading, hazy type than that produced by the hemolytic agent and it also occurs on plates containing serum as well as on the corpuscle plates. Thus under conditions that allow the culture to produce acid it may also cause an acid type of hemolysis which differs from the hemolysis produced by the hemolytic agent by being more diffuse, by changing the color of the blood to a brownish tinge, and by not being inhibited by serum. This type of hemolysis may also be demonstrated in fluid cultures by growing the culture in glucose bouillon for twenty-four hours and then testing with and without serum. Both tubes produce hemolysis and in contrast to the plain bouillon culture the blood shows a brownish color and the serum fails to prevent the reaction.

Before the writing of this report had been finished some further tests with 1192 *b* showed that this culture had lost its hemolytic property. A transplant from the stock *b* culture made now about one year from the beginning of the hemolytic studies produced little or no hemolysis. On the other hand, the stock *a* culture still caused complete hemolysis. If the *a* type was plated out on agar and *b* colonies or translucent areas of *a* colonies picked off and tested for hemolysis they were found to give a positive reaction. Thus there are produced two *b* variants, one hemolytic and one non-hemolytic.

## SUMMARY AND CONCLUSIONS

Among 14 bovine strains of *B. coli* two showed hemolytic properties. Strain 1192 was studied in more or less detail in both fluid and solid media. The experiments showed that the hemolytic agent is a product of the living culture and not an end product of growth since filtrates and killed cultures are non-hemolytic. The hemolytic agent is closely associated with the life of the bacteria for death of the bacteria and destruction of hemolytic activity run parallel.

The activity of the hemolytic agent is greater in 4 hour than in 24 hour bouillon cultures and is also greater in bouillon cultures than in agar growths suspended in salt solution. In general the hemolytic activity corresponds with the number of living organisms but the parallelism is hidden if inhibiting factors are present.

The culture grown under anaerobic conditions still produces the hemolytic agent.

The H-ion concentration was found to affect the hemolytic activity only when it checked the growth of the culture.

The hemolytic agent is active at refrigerator temperature and the low titer of cultures kept in the refrigerator is apparently due to a reduced production of the hemolytic agent rather than to a loss of its activity.

The hemolytic agent may be formed from peptone alone, or from certain media containing no peptone such as veal infusion broth, but it is not formed in a culture fluid of salt solution and glucose.

The hemolytic action of a bouillon culture is affected by salt solution dilution. Dilution experiments with different lots of bouillon and with filtered bouillon suggest that an inhibiting factor is present in bouillon. Peptone in certain concentrations was also found to have an inhibiting effect on the hemolysis, and the inhibiting substance in bouillon was considered due to the added peptone apparently combined with some material in the veal infusion. Other substances found to interfere with the hemolytic activity of the culture were certain normal sera and cholesterol.

Specific immunization increased or originated the inhibiting

value of sera indicating that the hemolytic agent may possess antigenic properties.

In media which allowed the culture to produce acid an acid type of hemolysis occurred but this differed from the hemolysis due to the hemolytic agent by being more diffuse on blood agar plates, by changing the color of the blood to a brownish shade, and by not being inhibited by serum.

A non-hemolytic *b* variant was obtained from the *b* hemolytic type after its long cultivation. Three types of Culture 1192 now exist, *a* the original form, and two *b* variants, *b* hemolytic and *b* non-hemolytic.

#### REFERENCES

- DUDGEON, L. S., WORDLEY, E., AND BAWTREE, F. 1921-1922 Jour. Hyg., **20**, 137.  
KOLLE, W., AND VON WASSERMANN, A. 1913 Handbuch der pathogenen Mikroorganismen, Jena, **6**, 499.  
SMITH, T., AND ASSOCIATES 1927 Jour. Exper. Med., **46**, 123.

# EFFECT OF SERA ON THE HEMOLYTIC REACTION OF A PATHOGENIC BOVINE STRAIN OF *B. COLI*

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In this work the same hemolytic strain of *B. coli* (1192) was used that is described in the immediately preceding paper. In the literature on inhibition of hemolysis by sera, first in regard to *B. coli* hemolysis, Kolle and Wassermann (1913) mention Kayser's report that hemolysis by *B. coli* was prevented by dog, rabbit, and human sera. Dudgeon, Wordley and Bawtree (1921-1922) found that human serum in certain concentrations prevented hemolysis by their *B. coli* strains. By comparing the action of the serum with that of a 6 per cent gum solution which had little effect on hemolysis they concluded that the action of serum in preventing hemolysis was something apart from mere mechanical protection. There are other references to the inhibiting effect of sera on hemolysis produced by other organisms or substances. Weinberg and Otelesco (1921) stated that normal horse serum possessed an anti-hemolytic power against *B. proteus* and that it also exerted an anti-infecting power proportional to its anti-hemolytic properties. Cole (1914) noted an anti-hemolytic action of serum for pneumococcus hemotoxin and related it to the cholesterol or lipid content of serum. Braun and Shi-Tsing (1922) reported *B. proteus* hemolysis as inhibited by immune but not by normal sera. Ponder (1923-1924; 1925; 1927) studied saponin hemolysis and reported that it was inhibited by serum. From his experiments he concluded that it was the protein of the serum and to a less extent the cholesterol that were the inhibiting factors. He noted that horse and human serum had a greater inhibiting power than rabbit, guinea pig, cat or mouse serum. Also he considered

that the inhibiting process corresponded to an adsorption reaction.

In the present work the inhibiting power of serum on hemolysis was first noted when whole horse blood was used with the bouillon culture instead of washed corpuscles with the result that no hemolysis occurred. A series of tests were then made with normal sera of different animals. These sera were tested in combination with various corpuscles. For the tests the sera were inactivated for one-half hour at 56°C. and used in a 1:10 dilution.

TABLE 1  
*Effect of sera on hemolysis*

CULTURE	SERUM (INACTIVATED) 1:10 DILUTION	HEMOLYTIC REACTIONS WITH CORPUSCLES OF					
		Horse	Sheep	Cow	Rabbit	Guinea pig	Human
24 or 48 hour bouillon growth 1192 b	Horse 1	—	—	—	—	—	—
	Horse 2	—	—	—	—	—	—
	Cow 174	—	—	—	—	—	—
	Human 1	—	±	+	—	—	—
	Cat 3	—					
	Cat 1	+	++	++	+	+++	+
	Sheep 1	++	++	++	+	+++	+
	Swine 380	+++	+++	+++	++	++++	++
	Rabbit 984	C	C	+++	++++	++++	+++
	Guinea pig 3859	++++	++++	C	++++	C	
	Calf 1466	+++					
	Calf 1480	C					
1192 b	No serum	C	C	C	C	C	C
Bouillon + serum		—	—	—	—	—	—

The results are given in table 1. Horse, cow, and one cat serum completely prevented hemolysis. Human serum caused almost complete inhibition. Sheep, a second cat, and swine serum allowed some but not complete hemolysis, and guinea pig, rabbit, and calf sera caused little or no inhibition of hemolysis.

Since the hemolytic agent is associated with the growth of the culture an attempt was made to see if the horse serum checked the growth during the test period in any way such as to inhibit

hemolysis. Rabbit serum was compared with horse serum and plain bouillon used as a control. Colony counts were made at the beginning of the test and after two hours at 37°C. The results were negative, so that the inhibiting action of horse serum on hemolysis is not considered to be due to its power to check the growth of the bacilli during the test period.

It was thought that perhaps the cholesterol content of the serum had something to do with its inhibiting action, since cholesterol is known to inhibit certain kinds of hemolysis. First the inhibiting action of a 1:10 dilution of a cholesterol suspension containing 40 mgm. per 100 cc. was tested with the culture against various corpuscles. The results showed an inhibition of hemolysis by such a cholesterol suspension. However, by comparing some figures<sup>1</sup> for free cholesterol in sera with their inhibiting action on hemolysis, it is seen that the two results do not always agree. Thus, apparently cholesterol in the serum is not the cause of the different inhibiting values of various sera although it probably plays some part in the general inhibiting effect of sera.

Since Ponder (1923-1924) claimed that the protein of serum was the chief factor inhibiting hemolysis, horse serum deproteinized according to his method was tested for its effect on the hemolytic action of culture 1192. The serum was heated to coagulate the protein and then filtered. Such serum no longer prevented hemolysis.

Robertson (1912-1913) has recorded figures comparing horse and rabbit sera with respect to protein content which in terms of the per cent of total protein show 63 per cent globulin and 37 per cent albumin for normal horse serum, and 28 per cent globulin and 72 per cent albumin for normal rabbit serum. Since the protein of the horse serum evidently interferes with hemolysis, possibly the different protein composition in rabbit serum may explain its different behavior in regard to hemolysis. To demonstrate this chemical tests would be necessary, which were not made at this time.

However, the following experiments again compared two sera of

<sup>1</sup> The cholesterol values on the samples of serum used were determined by Dr. R. E. Shope.

different protein composition, namely, cow and calf serum, to see if they showed a difference in their power to inhibit hemolysis similar to that existing between horse and rabbit serum. Howe (1921) found that the serum of the newborn calf was lacking in certain proteins (euglobulin and pseudoglobulin I). He also stated that these proteins might be absent from the blood of older calves but so far as he had examined the blood of adult animals the serum always contained these globulins. When calf and cow sera were compared for inhibiting power on hemolysis the results showed that calf serum possessed little or no power to prevent hemolysis, while cow serum completely inhibited hemolysis in a 1:10 to 1:40 dilution which corresponds with the differences obtained between rabbit and horse serum. Also, cow serum deproteinized according to Ponder's method lost its power to prevent hemolysis.

Finally milk was taken as another protein substance to be compared with serum for inhibiting power on hemolysis. Fat-free milk as prepared for media and also fresh milk separated from the cream by centrifuging failed to prevent hemolysis. These experiments show a certain relation between the inhibiting power of sera and their protein composition, since the milk and also the sera (calf and rabbit) that failed to inhibit hemolysis were lacking in certain globulins or contained a lower per cent of globulin than the sera which prevented hemolysis. Therefore it may be inferred that the different protein compositions of various sera account for their different inhibiting values, and possibly the amount of globulin or the ratio of globulin to albumin in the serum is a factor in its inhibiting reaction.

Experiments were also made to see if any parallelism existed between immunity reactions of sera and their power to inhibit hemolysis. The agglutination test was used to indicate the immune properties of the sera. First sera from an untreated normal horse and rabbit were compared for agglutinin content and anti-hemolytic power. The results showed that the horse serum agglutinated Strain 1192 to a titer of 1:1,280 and completely inhibited hemolysis in a 1:10 dilution, while rabbit serum showed an agglutination titer of 1:160 and still allowed good

hemolysis in a 1:1 dilution of the serum. When these experiments were extended to cow sera it was found that 8 normal sera inhibited hemolysis in 1:10 to 1:40 dilutions and gave an agglutination titer of 1:320 to 1:640. The results with three of these sera are recorded in table 2. The sera of 2 cows immunized with 1192, on the other hand, gave an agglutination titer of 1:5,120 and the serum of the cow treated with killed culture caused inhibition of hemolysis in a 1:80 dilution while the serum from the cow immunized with living culture inhibited hemolysis in 1:320 to 1:640 dilutions. Again serum from a rabbit was tested before and after immunization with 1192 (table 2). Normally the serum gave an agglutination titer of 1:160 and almost no inhibition of hemolysis. After treatment with killed culture the agglutination titer was 1:5,120 and a 1:5 dilution of the serum caused nearly complete inhibition of hemolysis. After continued immunization with living culture the agglutinin titer rose to 1:40,960 and the serum prevented hemolysis in a 1:40 or 1:80 dilution.

Absorption experiments with horse and cow sera showed that complete absorption of agglutinins caused only a slight decrease in the anti-hemolytic power of these sera. This may be explained by considering that the anti-hemolytic power is connected with the immunity reaction on the basis that immunization increases the anti-hemolytic power by increasing the globulin (protein) content of the serum rather than by simply producing a specific antibody for the hemolytic agent. Immunization is known sometimes to increase the globulin content of a serum and absorption of antibodies probably would not cause an equal reduction of globulin content. Therefore these absorption results may be interpreted in favor of the theory that the inhibiting factor of serum is connected with the globulin and the increased inhibiting power of immune serum is due to its increased globulin content. However, there is also some indication of a production of specific anti-hemolysin. This is shown most strongly in the case of an immunized rabbit. The absorption experiment with this rabbit serum definitely indicated a specific anti-hemolysin. The normal serum caused little or no inhibition of hemolysis and agglutinated the culture in a 1:160 dilution. The immune



TABLE 2  
Comparison of antibody content and hemolysis inhibiting power of sera

I. AGGLUTINATION													II. HEMOLYTIC TESTS											
Culture	Serum	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	S. S. control	Culture	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Culture control	Corpuscle control
1192 24 hour bouillon growth diluted 1:1 with salt solution	Normal cow 1121	++	++	++	++	++	+	+	+	+		-	1192 4 hour bouil- lon growth	-	-	-	-	+	+	+	+	+	+	+
	1134	++	++	++	++	++	+	+	+	+					-	-	+	+	+	+	+	+	+	+
	1229	++	++	++	++	++	+	+	+	+					+	+	+	+	+	+	+	+	+	+
	Immune cow 1374 (killed culture)	+	+	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+	+
1231 (living culture)	1231 (living culture)	+	+	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+	+
	Normal rabbit 1019	++	++	++	++	++	+	+	+	+					+	+	+	+	+	+	+	+	+	+
1019 (killed culture)	Immune rabbit 1019 (killed culture)	+	+	+	+	+	+	+	+	+					+	+	+	+	+	+	+	+	+	+
	Immune rabbit 1019 (living culture)	++	++	++	++	++	++	++	++	++					+	+	+	+	+	+	+	+	+	+

TABLE 3  
Evidence of anti-hemolytic antibody production. (Rabbit serum)

		I. AGGLUTINATION										II. HEMOLYTIC TESTS									
Culture 1192 b	Serum	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	S. S. control	Culture 1192 b	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Corpuscle control
24 hour bouillon growth	Normal	++	+	-	-						-	4 hour bouillon growth	C	C	C	C	C	C			
	Immune (killed culture)	++	++	++	++	++	++	++	+		-		+	+	+	C	C	C			
	Immune (killed culture) absorbed by 1192	-	-	-	-	-	-	-	-		-		C	C	C	C	C	C			
	Immune (living culture)	C	C	C	C	C	C	++	++	++	-		-	-	-	-	-	+	++	++	
	Immune (living culture) absorbed by 1192	++	++	++	++	++	++	++	++	+	-		+	+	+	++	++	++	C	++	
		++	++	++	++	++	++	++	++		-		+	+	+	++	++	++	C	++	

serum, produced with killed culture, allowed only a one plus (+) hemolysis in a 1:5 dilution and gave an agglutination titer of 1:5,120. Absorption of the serum by 1192 reduced the agglutinin titer to 1:20 and again allowed complete hemolysis in a 1:5 dilution (table 3). Furthermore absorption of the immune serum obtained after continued immunization with living culture reduced the agglutination titer from 1:40,920 to 1:2,580 and the anti-hemolytic power from complete inhibition of hemolysis by a 1:80 dilution to partial inhibition of hemolysis in a 1:5 dilution.

#### SUMMARY AND CONCLUSIONS

Certain normal sera (horse, cow and human) prevent hemolysis by *B. coli* 1192 and other sera cause less inhibition or practically none (especially rabbit, guinea pig and calf sera).

Cholesterol in certain concentrations prevents hemolysis but cholesterol in serum is not considered the explanation of the different inhibiting values of different sera on hemolysis since cholesterol content and inhibiting power do not always agree. However, cholesterol in the serum probably exerts some influence on the general inhibiting effect of sera on hemolysis.

Apparently the protein of horse and cow serum is an inhibiting factor. Rabbit and horse serum have a different protein composition which may explain their different inhibiting powers. Calf and cow serum are also of different protein composition and show correspondingly different inhibiting effects on hemolysis. Milk differs from cow serum in failing to prevent hemolysis. These results lead to the inference that the protein composition of serum apparently affects its behavior toward hemolysis and the amount of globulin or ratio of globulin to albumin is possibly a factor in the reaction.

The inhibiting action of serum is increased by specific immunization. This seems to be due, at least in part, to specific antibody formation, and also it may be the result of an increased globulin content in the immune serum.

Apparently the inhibiting power on hemolysis of any given serum may be due to a combination of factors including cholesterol, protein composition and specific antibody content; but the

different inhibiting values of different normal sera seem to be related chiefly to their protein composition.

## REFERENCES

- BRAUN, H., AND SHI-TSING, C. 1922 *Centralbl. f. Bakt., I Abt., Orig.*, **89**, 139.  
COLE, R. 1914 *Jour. Exper. Med.*, **20**, 346.  
DUDGEON, L. S., WORDLEY, E., AND BAWTREE, F. 1921-1922 *Jour. Hyg.*, **20**, 137.  
HOWE, P. E. 1921 *Jour. Biol. Chem.*, **49**, 115.  
KATSER, H. In Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, 1913, **2**, 1358, Jena.  
PONDER, E. 1923-1924 *Proc. Roy. Soc., London*, **95**, 42.  
PONDER, E. 1925 *Proc. Roy. Soc., London*, **98**, 484.  
PONDER, E., AND McLACHLAN 1927 *British Jour. Exper. Path.*, **8**, 267.  
ROBERTSON, T. B. 1912-1913 *Jour. Biol. Chem.*, **13**, 325.  
WEINBERG, M., AND OTELESCO, I. 1921 *Compt. rend. Soc. Biol.*, **84**, 535.



## CONCERNING THE MANUAL OF METHODS PUBLISHED BY THE SOCIETY

### REPORT OF COMMITTEE ON BACTERIOLOGICAL TECHNIC<sup>1</sup>

For something over twenty years the Society of American Bacteriologists has been publishing a Descriptive Chart for the identification of bacteria. This chart has been revised from time to time and the latest revision has always been on sale by the society; the sale was handled by the secretary in former years, and more recently by the chairman of this committee. The object of this chart has been quite generally understood—namely, a means for the standardized description of cultures. It has been used to some extent by research workers as a means for filing a record of the cultures they have studied, but to far greater extent in instruction laboratories to give students an idea of the methods by which cultures may be described and identified.

The first editions of this chart were fairly simple and the methods for making the determinations were either self-explanatory or else were stated on the chart itself. As the chart expanded and the bacteriological technic became more complicated, it was no longer so easy to decide what technic to employ in making the determinations called for on the chart. It was accordingly suggested to the committee in 1913 or 1914 that they prepare a manual of methods to be used with the chart. It was a few years later before a beginning was made in this line, but finally in 1918 a report on methods was printed in the *JOURNAL OF BACTERIOLOGY*, which was put on sale in reprint form as a manual to accompany the chart. This report was revised in 1920.

In 1923 a more pretentious manual was issued in loose-leaf form. This is the present Manual of Methods for Pure Culture

<sup>1</sup>H. J. Conn, Geneva, N. Y., Chairman; Victor Burke, Barnett Cohen, Elizabeth F. Genung, W. L. Kulp, and W. H. Wright.

Study of Bacteria. In connection with this Manual, the Committee maintains a continuation service to which any owner of a copy of the Manual may subscribe if he wishes to keep his copy up-to-date. Sections of the publication are revised from time to time and sent to the subscribers.

The object of this Manual at the start was very plainly the same as that of the shorter reports which had preceded it, namely to serve as a guide for use with the Descriptive Chart in the identification of species. This object of the Manual is not so well understood as that of the Chart. There seems to be a common impression that the Committee has intended the Manual as a general handbook on bacteriological technic. This impression has become more pronounced in recent years, partly because the Manual has found its way to libraries and to many laboratories where the descriptive chart is not used, and partly because recently several supplements have been added to the Manual which have considerably broadened its scope.

It was desired to learn somewhat how widespread among the users of the Manual the impression had become that it was intended to serve as a general laboratory guide in bacteriology. It was also desired to find out whether the users of the Manual would prefer to have the publication assume more nearly its original aspect, or on the other hand, to be broadened still further in its scope so as to become more frankly a handbook in general bacteriological technic. Accordingly a questionnaire was sent out early this year to the Manual owners who had subscribed to its continuation service. Part of this questionnaire related to the descriptive chart and a discussion of that part will be saved for another occasion. Four of the questions, however, concerned the matters just mentioned; these questions with the replies received follow:

1. This Manual was originally intended merely as a handbook to accompany the Descriptive Chart, giving the methods to be used with the latter. Recent supplements are tending to broaden its scope. Shall we continue the policy of broadening the field covered?

2. Would you prefer to return definitely to the original policy of

limiting its field to the methods needed in studying bacteria by means of the Descriptive Chart?

These two questions are closely related and an affirmative answer to the first required a negative answer (or no reply) to the second. The replies to these two questions must therefore be listed together. Seventy-two answered the first question in the affirmative, sixty-one of whom replied "no" to the second question, while the others in making no reply to the latter evidently assumed that this was fully answered in their affirmative reply to question one. Four answered "no" to question 1, and replied to the second question in the affirmative. In one other instance, the reply to question 2 was: "Yes, in a way" and that to question 1: "See no objection to moderate amount of such material, but would guard against the possibility of making it unwieldy. Better stick to material for chart." In addition to those just referred to there were eight who failed to reply to either question.

3. If the policy of broadening the scope is to be continued what kinds of methods should be included?

This question could not be answered categorically; but more than half of those replying took this opportunity to make some suggestions. A number of these suggestions summed up in a few words are as follows:<sup>2</sup> formulae for special media (7); all *standard* methods (4); more on serology (3); more staining methods (2); methods of enumeration (2); more on fermentation (2); diagnostic procedures (2); methods for anaerobes (2); technic for filterable viruses (2); disinfectant coefficient tests (2); "all practical methods" (2); all laboratory methods; any methods found useful in technical and manufacturing bacteriology; methods for bacteriophage; technic for yeasts and fungi; electrometric H-ion methods; technic for studying morphologic variations; methods for single cell culture, for studying microcobic dissociation and for determining filterability of micro-

<sup>2</sup> The figures in parenthesis indicate the number of replies specifying each item listed. When no figure occurs it is to be understood that but one such reply was received.



organisms; methods for testing pathogenicity; procedures for determining metabolic products; technic for isolating and maintaining pure cultures; methods for studying enzymes; methods for oxidation and reduction, surface tension, etc.; analytical procedures for water, milk, food and soil.

4. In that case, is the present title of the Manual sufficiently descriptive? If not, what title would be more suitable?

This question was one on which fewer wished to express an opinion, as forty-five of those returning the questionnaire did not make any reply. Thirty expressed themselves as satisfied with the present title while ten replied in the negative. The following suggestions for titles were sent in:<sup>1</sup> Manual of Laboratory Methods (2); Manual of Methods for Pure Culture Study and General Technic; Manual of Approved Methods of the S. A. B.; Methods in Isolation and Pure Culture Study; Manual of Bacteriological and Serological Technic; Standard Methods; Manual of Laboratory Directions; Manual of Methods for Culture Study of Bacteria; Manual of Methods for Study of Bacteria.

One thing is very plain from the replies that were received. There is no question but that those of the subscribers to the Manual continuation service, who were interested enough to reply to this questionnaire, are in general anxious to see the scope of the Manual enlarged and will be glad to see methods in almost any line of bacteriology added to it. It must be remembered, however, that the subscription list for the Manual continuation service is something less than 400 and that of the questionnaires sent out, only 85 were returned. Obviously these replies represent a very small proportion of the membership of the Society, and the list to which the questionnaire was sent was a selective one, composed of bacteriologists likely to be especially favorable to the expansion to the Manual. It can not, therefore, be assumed that this reply, even though overwhelmingly in favor of a policy of expansion, gives the committee any mandate to adopt such a policy.

Before adopting such a policy it must be learned whether the Society in general will approve of letting a publication issued by

one of its committees assume the character of a manual in general bacteriological technic. The committee, moreover, has always been opposed to standardization of methods except in the case of control work, while if the Manual assumes the character suggested by the replies above referred to, it might soon become generally known as a manual of "standard methods" even though it were specifically stated by the committee that the methods were *not* to be considered official or standard. It is very plain that this is a matter of policy that the committee itself cannot decide, but it must be discussed as widely as possible by the Society membership. It will be brought up at the next meeting of the society. In the meantime the committee chairman will be glad to hear by letter from anyone interested in the subject.

The subject calls for a careful consideration. There is abundant evidence that the Manual is proving useful. Its sale continues at the rate of a few hundred each year and the subscription list to the continuation service is continually growing with very few failures to renew expired subscriptions. A large number of those replying to this questionnaire made special reference to the value of two important features of the Manual: first, that the methods included in it have actually been tried out in laboratory use and are not merely quoted from some textbook; second, that the loose-leaf form of publication makes it possible to maintain the Manual constantly up-to-date and to keep its subscribers in touch with the latest developments in technic. That a similar undertaking with a broader scope might be made even more valuable, if properly handled, is quite evident. The committee, however, is not willing to commit itself as to whether the present time is ripe for such an undertaking, or as to whether the present membership of the committee is sufficiently representative of bacteriology in general to handle it successfully. Comments on the subject, therefore, whether favorable or unfavorable, will be very welcome.



# THE INFLUENCE OF PUTREFACTIVE GASES ON B. ANTHRACIS

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An interesting communication by Valley and Rettger (1927) dealing with the influence of carbon dioxide on different bacteria under various conditions has impelled me to publish the results of the experiments described below. These experiments, which have now been carried on for a considerable period, were stimulated by the practical necessity for stimulating the spore formation of the anthrax bacillus under certain conditions and for suppressing it under others.

The need for stimulation of sporulation arose because in Russia the spore vaccine of Cenkowski is generally used. The spore "Matrix" of these vaccines (I and II) has been maintained since the year 1886 in the Veterinary Bacteriological Institute at Kharkov, and has been distributed to the various Russian laboratories preparing the vaccine for practical use. Owing to the great distances in Russia the excessive heat in summer and the difficult conditions of transportation<sup>1</sup> only spore vaccines could be widely used (about one and a half million animals are thus vaccinated yearly).

Naturally all the Russian laboratories attempted to produce vaccines containing a maximum proportion of spores and finally the traditional Pasteur method of cultivating anthrax vaccines in broth was replaced by cultivation on agar plates.<sup>2</sup> By the very simple technique for this mode of vaccine preparation elaborated

<sup>1</sup> A package of vaccine is often sent by "horse-post" for 1000 kilometers or more.

<sup>2</sup> The traditional glycerol solution was replaced by a physiological solution of sodium chloride.

by Vyshelesski (1909), the sporulation was much more complete than in broth, but even with this procedure and under the best conditions of temperature, aeration, etc., nearly 20 per cent of the organisms were still in the vegetative state. We desired therefore to improve the method further so as to obtain a still higher proportion of spores. Among many such attempts made during my term of office as director of the Anthrax Laboratory in the Veterinary Bacteriological Institute at Petrograd (1911-1912) the most interesting seem to be the efforts of Michajlowski (1926) to stimulate the sporulation of *B. anthracis* by exposure to the vapors of certain volatile substances. By microscopic examination and by experiments on laboratory animals it has been estimated that after exposure to the vapors named in the work of Michajlowski it is difficult to find in agar culture any vegetative cells of the bacillus, while the original virulence of the culture is not affected.<sup>3</sup>

It is now planned to conduct systematic experiments designed to explain in detail the influence of pH and of insufficiency of nutriment upon the sporulation of *B. anthracis*.

The desire on the other hand to suppress the sporulation of *B. anthracis* has come from the necessity for checking the spread of anthrax in pasture lands. The disease is now extending in many parts of Russia chiefly from the bodies of animals which have died of the infection. Our guiding principle in these attempts was the familiar observation that during the putrescence of animal cadavers all the vegetative forms of *B. anthracis* are quickly destroyed and only those spores from the skin, the excrement, etc., remaining above the surface of the earth produce subsequent infection. To make practical use of this fact it was necessary in the first place to determine exactly which products of putrefaction were effective in destroying the bacillus. In the thesis by Velicky (1923) the first preliminary attempts were made to discover whether this action is due to non-volatile substances

<sup>3</sup> Each of the series of anthrax vaccines in the Petrograd laboratory was tested for its effect on animals before sending it out for practical use. The first vaccine killed all white mice, and left all guinea pigs alive. The second killed all guinea pigs and left all rabbits alive.

or to gases of putrefaction. The attempts to explain the action of the non-volatile substances were technically very simple. From thoroughly putrefied animal organs, extracts were made following the usual procedure for the preparation of meat extracts from microbial cultures. During the process of boiling for two hours, of hot filtration and of sterilization in the autoclave, all the volatile substances present in the putrefied organs were necessarily driven off and only non-volatile substances soluble in water remained. It was found that in these extracts from putrefied organs at a pH of 7.0 to 7.5, the anthrax bacillus was not only not killed but multiplied very well even without the addition of

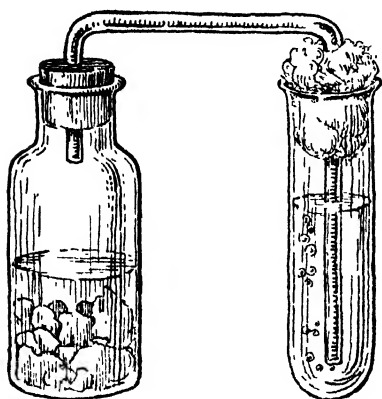


FIG. 1

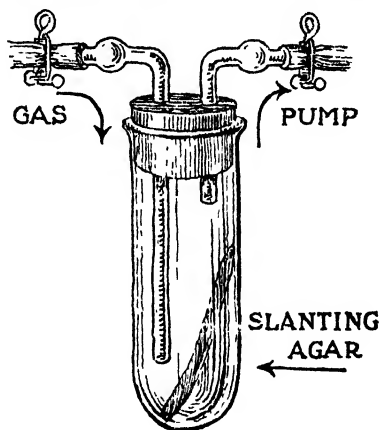


FIG. 2

1 per cent peptone. Therefore we can conclude that non-volatile substances do not play a part in the destruction of the anthrax bacillus in putrefying cadavers.

The attempt to establish the action of the gases of putrefaction was made in the following way. A bottle was half filled with cattle meat and parts of organs, with the addition of a sufficient amount of water and the bottle was closed with a rubber cork perforated by a bent glass tube. After putrefaction had set in the free end of the glass tube was sterilized in the Bunsen flame and pushed to the bottom of a test tube containing a broth culture of the anthrax bacillus as shown in figure 1. After exposure for sixteen to twenty-four hours to the action of the gases generated

in the bottle as they bubble through the broth culture, all the bacilli and all the spores of the anthrax bacilli were killed as shown by culture methods and injection into laboratory animals.

Our next object was to determine which of the particular putrefactive gases exert destructive action. The following gases known to be the principal gases produced by putrefaction were tested:  $\text{CO}_2$ ,  $\text{H}_2$ ,  $\text{CH}_4$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{S}$ . These tests were made in the following fashion. A large test tube with a rubber cork perforated by two glass tubes and containing a slant agar culture was used (see fig. 2). By repeated exhaustion with a pump, a pure atmosphere of the gas studied was obtained in the tube over

TABLE 1

GASES	AGAR FRESHLY INOCULATED		GROWTH ALREADY DEVELOPED		RESULTS OF REINOCULATION INTO ANIMALS
	Growth	Spore formation	Growth	Spore formation	
$\text{H}_2$ .....	+	+	+	+	+
$\text{CH}_4$ .....	+	+	+	+	+
$\text{NH}_3$ .....	±	±	-?	-?	+
$\text{CO}_2$ .....	±	+	±	+	+
$\text{H}_2\text{S}$ .....	-	-	-	-	-

± signifies that only 1 or a very few small colonies appeared, or in other words few spores had survived.

the agar. The various gases were allowed to act either on agar slants just inoculated or on agar slants on which the bacteria had already developed. The results are indicated in table 1.

These experiments indicate that of the gases tested hydrogen sulphide has the most powerful destructive effect on *B. anthracis* and quickly kills not only the vegetative cells but the spores. This gas can be very simply and cheaply prepared in large quantities by treating sulphide of iron with an acid. Several experiments were therefore made to test the practical applicability of hydrogen sulphide for the treatment of the bodies of animals dead from anthrax in the following way. The cadavers of guinea pigs dead from anthrax were put into a large glass cylinder and sprinkled with sulphide of iron; dilute hydrochloric acid was

poured over them and they were momentarily covered with earth. The energetic formation of bubbles of hydrogen sulphide was apparent and attempts made a week later at cultivation of the organism and inoculations of animals all proved negative. These results permit us to conclude that even though all bacilli and spores may not be killed by such a treatment of a cadaver with hydrogen sulphide their numbers are so greatly reduced as to be of substantial value in decreasing the danger of spread of the disease.

## REFERENCES

- MICHAJLOWSKI 1926 *Centralbl. f. Bakt., Orig.*, **97**, 17.  
VALLEY AND RETTGER 1927 *Jour. Bact.*, **14**, 101.  
VELICKY 1923 The influence of non-volatile substances and gases of putrefaction on *B. anthracis*. Brunn, Czechoslovakia.  
VYSHELESSKI, S. N. 1909 The cultivation and application of anthrax-agar-vaccines. Petrograd.





# THE PRECIPITATION OF MAGNESIUM AMMONIUM PHOSPHATE CRYSTALS DURING THE GROWTH OF BACTERIA IN MEDIA CONTAINING NITROGENOUS SUBSTANCES

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In October, 1924, minute crystals were noted in veal infusion ascitic agar, upon which gonococci were growing. The crystals, which were visible to the naked eye two to four days after inoculation, had the microscopic morphology of the triple phosphates commonly found in alkaline urine. Similar crystals were found beneath the layer of growth in tubes of semi-solid hormone agar containing stock cultures of gonococcus, meningococcus, and *Micrococcus catarrhalis*.

## REVIEW OF LITERATURE

Previous to my abstract in January, 1926, no reference had been made in the literature to the association of ammonium magnesium phosphate crystals with viable bacterial growth in hormone media, rich in organic nitrogen and phosphorus. The presence of similar crystals was reported by Arzuni (1890) in peptonized meat, by Palache (1923) in canned shrimp, by Clark and Clough (1925) in canned salmon, and by Porter (1924) in the lungs of a cadaver. Laidlaw (1925) noted structures with the appearance of microscopic colonies, which he attributed to the separation of calcium and magnesium soaps from the uninoculated medium. Recently Huddleston and Winter (1927) identified magnesium ammonium phosphate crystals in cultures of *B. abortus* and *B. melitensis*. They associated the rate of crystal formation with the initial reaction of the culture medium, the rate of growth, and

the viability of the organisms. Pillet (1927) found that staphylococci, incubated in aseptic acid urine, produced an alkaline reaction and caused precipitation of ammonium magnesium phosphate crystals, whereas the colon bacillus retarded or prevented this precipitation.

#### METHOD OF STUDY

Since the original observation, records of the occurrence of these crystals have been kept and the influence of various media and procedures upon their formation has been studied in sixty-five strains of bacteria.

The medium of preference was a beef-heart hormone base with 0.5 per cent agar, 1 per cent peptone, bromthymol blue indicator and adjustment to pH 7.0 with 10 per cent sodium carbonate. It was poured to about one-half the depth of the tubes, which measured 4 by  $\frac{3}{8}$  and 6 by  $\frac{1}{2}$  inches respectively. The ingredients of the medium were varied by the addition of egg, ascitic fluid, peptone, sodium chloride, sodium carbonate, sodium hydroxide, and excess water. In order to eliminate the meat infusion base, the synthetic medium described by Enlows (1923) which contained peptone, 0.5 per cent agar, water and potassium monohydrogen phosphate was substituted. All fecal organisms grew well on the synthetic media, especially in the presence of fermentable carbohydrates.

#### OBSERVATIONS

Our observations on inoculated and uninoculated media were only qualitative. The bacteria fell into certain groups according to the precipitation of ammonium magnesium phosphate in the presence of meat infusion, peptone, and agar. The two strains which produced crystals most readily belonged to the heterogeneous group of *B. alcaligenes* and possessed the property of liquefying gelatin. Usually the crystals first appeared in the zone of growth and later settled out into the agar. Frequently they appeared along the stab even when visible growth did not occur. In one instance an old culture of *Actinomyces hominis* produced the crystals in meat infusion broth.

The progress of crystal precipitation followed closely changes in the hydrogen-ion concentration of media as indicated by the color changes in the bromthymol blue indicator. The optimum initial hydrogen-ion concentration of the medium was between 6.9 and 7.1 pH. Organisms such as streptococci and pneumococci which produce a permanent acidity and ferment the ordinary carbohydrates formed no crystals. Organisms, such as *B. alcaligenes*, which cause permanent alkalinity and do not ferment the ordinary carbohydrates brought about rapid precipitation. Closely allied strains varied in their ability to produce crystals.

No difference was observed when the reaction of the media was adjusted with sodium hydroxide or sodium carbonate. An initial acid reaction retarded precipitation of crystals. For instance, unadjusted acid infusion agar gave rise to the crystals with favorable strains of bacteria after a long incubation period. An initial alkaline reaction favored the production of crystals. Uninoculated alkaline media did not produce crystals upon standing, although an occasional crystal was observed in tubes of media made sugar free with *B. coli*, to which raffinose and dulcitol had been added.

The ingredients of the media caused differences in crystal formation. Excess water retarded precipitation. Sodium chloride, 0.5 per cent, retarded the formation of crystals, which were produced slowly in media with salt by favorable strains and either not at all or after several weeks by the less active crystal-producing organisms.

With a meat infusion base the addition of peptone, egg, and ascitic fluid had no marked influence upon the precipitation of crystals. One per cent peptone favored the production of crystals. In synthetic media consisting of potassium monohydrogen phosphate, 0.5 per cent peptone, and agar, minute fine glistening crystals developed with the bacteria which produced crystals most readily. These same bacteria produced in meat infusion hormone media more numerous and larger crystals of a feathery appearance.

## CRYSTALS

The first crystals were separated from seven-day-old cultures grown in semi-solid agar by melting the agar, washing in distilled water, and holding in 95 per cent ethyl alcohol until examined. Later a single crystal was extracted from each of ten cultures of *B. alcaligenes*, *M. catarrhalis*, and *C. diphtheriae*. The crystals were teased free from the media on a glass slide, sterilized with 5 per cent phenol, and washed with ethyl alcohol. The identification of the crystals was made by measuring their optical constants, especially their several indices of refraction with a polarizing petrographic microscope.

Examination of the crystals separated from the melted agar revealed the fact that they were mixtures of two and three compounds. Compound 1 had the optical properties which corresponded to those of  $\text{NH}_4\text{MgOP}_2\text{O}_6$  and Compound 2, those of  $\text{K}_2\text{Mg}_2(\text{SO}_4)_3$ . Compound 3 was an unidentified, anisotropic compound which occurred as a minute fibrous aggregate too intergrown with the above compounds to permit satisfactory optical determination.

The arrangement of these compounds within the "crystals" affords a key to their mode of formation. No. 1 always occurs in the center of crystals or is entirely absent. No. 3 occurs as almost cryptocrystalline clusters surrounding and penetrating no. 1, if the latter is present, No. 2 molds itself about these clusters and gives the resulting crystal form.

The optical properties of all the single crystals isolated from the different cultures identified them as  $\text{NH}_4\text{MgPO}_4 \cdot 6\text{H}_2\text{O}$ . Under the microscope the crystals displayed the typical orthorhombic, hemicorpic symmetry, and twinning characteristic of this compound and the crystallography similarly corresponded. Positive phosphate tests, together with the optical and crystallographic data leave no doubt as to the identification. These optical properties are:

Optical character.....	biaxial +
Optic axial angle.....	$2V = 30^\circ$ approximately
Dispersion.....	p (V) strong

Optic plane.....	parallel to <i>a</i> (100)
Optical orientation.....	<i>z</i> = <i>b</i>
	<i>X</i> = <i>c</i>
Indices .....	<i>d</i> = 1.493 - 0.002
	<i>b</i> = 1.496 - 0.002
	<i>s</i> = 1.504 - 0.002

## DISCUSSION

The significance of ammonium magnesium phosphate crystal production by bacteria does not appear of importance at the present time. Under properly controlled conditions with synthetic media of known composition the rate of crystal production of ammonium magnesium phosphate crystals may constitute a measure of bacteria utilization of amino acids with liberation of ammonia.

## SUMMARY

Bacteria vary in the formation of ammonium magnesium phosphate crystals in nitrogenous media. Favorable factors for crystal production are the presence of meat infusion, peptone and inorganic phosphate, decrease in sodium chloride and moisture. A medium containing peptone and phosphate without hormone infusion is less favorable for the precipitation of the crystals.

Acknowledgment is herewith made to Mr. M. J. Buerger of the Massachusetts Institute of Technology for his identification of the crystals.

## REFERENCES

- ARZUNI. *Zeit f. Kryst*, 1890, **18**, 60.  
 CLARK, E. D., AND CLOUGH, R. W. *Pacific Fisherman*, 1925, **23**, 11.  
 ENLWS, ELLA M. A. Reprint No. 868, Public Health Reports, September 14, 1923.  
 HUDDLESTON, I. F., AND WINTER, O. B. *Jour. Infect. Dis.*, 1927, **40**, 476.  
 LAIDLAW, P. P. *Brit. Jour. Exp. Path.*, 1925, **6**, 36.  
 PALACHE, C. *Jour. Min. Soc. of America*, 1923, **8**, 72.  
 PILLET. *Jour. d'urol., med. et chir.*, 1927, **24**, 5.  
 PORTER, M. W. *The American Mineralogist*, 1924, **9**, 93.  
 SCUDDER, S. A. Abstract, *Jour. Bact.*, 1926, **11**, 102.



# THE FERMENTATION OF GLUCOSE BY ORGANISMS OF THE GENUS *SERRATIA*

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## INTRODUCTION

The purpose of this paper is to discuss the biochemical changes produced in the fermentation of glucose by certain red bacteria of the genus *Serratia* Bizio, and to relate these changes to previous work with species of other genera, in an attempt to establish the relationship of this group to other groups. The cultures studied, *S. marcescens* Bizio, *S. indica* Bergey, and two additional closely related types that produce visible gas in Smith tubes from broth containing glucose, have been selected from about 200 cultures gathered from all parts of the world in the course of a monographic study of the genus.

## DESCRIPTION OF THE CULTURES

In a recent paper, Breed and Breed (1927) have redefined this genus so as to limit it to "the Gram-negative, non-spore-forming, nitrate reducing, gelatin liquefying, usually motile rods which produce the characteristic pigment 'prodigiosin.'" They also state that this complex pigment cannot be sharply defined at present; but that it varies from a bright orange-red on alkaline media and in fresh cultures to a dark magenta red on acid media or in old cultures. It is not produced under anaerobic conditions. Metallic, fuchsin-like effects may be constantly or intermittently produced. The pigment is readily soluble in alcohol. These bacteria usually produce acetyl methyl carbinol abundantly. Some strains, however, do it only intermittently or under special conditions and might readily be classed as acetyl methyl carbinol



negative if not tested under the proper conditions. The fermentation of sugars and the end products of fermentation are much the same as in the colon-aerogenes group. Usually, no visible gas is produced in fermentation tubes; but certain cultures produce gas regularly from glucose and other sugars. These organisms show motility best in sugar broths. Usually four peritrichous flagella are present. They grow best at 20° to 25°C. and either fail to grow or grow poorly at 37°C. If inoculated on agar slants already warmed to this temperature, some species die out in twenty-four to forty-eight hours. Other species are able to live longer than this though none are able to produce pigment abundantly at this temperature.

When the genus is limited in this way, it becomes evident that non-pigmented strains of these organisms show only minor differences from *Aerobacter aerogenes* Beijerinck and *A. cloacae* Bergey as defined by Weldin (1927).

The history of the six cultures used in this investigation is as follows:

*Serratia marcescens*. Culture 1377 was secured from the National Collection of Type Cultures of the Lister Institute, London, England, November 2, 1923, and carried the label "*Bacillus prodigiosus* from Delft." Our investigation of the culture shows it to be a typical well pigmented strain of this organism.

Culture HY was secured from Dr. L. F. Rettger of New Haven, Conn., October 23, 1923, and was obtained by him from the American Museum of Natural History Collection, New York City, five or six years previously. It has thus far proved impossible to establish the identity of this culture more exactly as several strains of *Bacillus prodigiosus* were carried in this collection. This culture has been used in investigational work done during the past ten years in Dr. Rettger's laboratory and is a strain of *S. marcescens* which has a tendency to produce white strains. In a fresh transfer it frequently grows in a mosaic of red and white.

*Serratia indica*. Culture K5 was secured from the Kral Collection in Vienna, Austria, February 19, 1924. It bore the label

"*Bacillus ruber indicus*" and the notation that it was the organism isolated by Koch in India from the digestive tract of an ape, and that it had been sent by Koch to the Kral Collection, January 3, 1904. This strain appears to be entirely typical though earlier descriptions indicate that it may at one time have been more heavily pigmented than it is at present.

Culture CU37 was secured from Cornell University, Department of Dairy Industry, October 27, 1923. This culture was isolated by Dr. W. A. Whiting from the rinsings of a milk can at Ithaca, N. Y., in connection with the investigation reported in Tech. Bull. No. 98 of the New York State Agricultural Experiment Station at Geneva, N. Y. Like Koch's original strain, it appeared as a single culture on a plate and was saved because of its striking chromogenesis. The culture was thought to be *Bacillus prodigiosus*, but the investigations of Breed and Breed (1926) show it to be a typical culture of *S. indica*. At present it is no more heavily pigmented than Koch's original strain.

*Serratia* sp. Culture K6 clearly does not belong to either of the above species, nor is it evident what name should be applied to it. It was received from the Kral collection, December 26, 1923 and bore the label "*B. rubrum*, Kiel (*B. balticum*)," names that would indicate that it was a sub-culture of Breunig's (1888) original culture isolated in 1888 from tap water in Kiel Germany and widely distributed to bacteriological laboratories during the following decade. This Kral culture, however, bore the notation "Isolated by Lubinsky, Inst. Pasteur, Kiev" and a reference to the catalogue of the Kral collection issued in 1919 indicates that this culture is not a sub-culture of the original. This strain is not heavily pigmented at present.

Meanwhile we obtained from Dr. F. G. Novy of Ann Arbor, Michigan, a sub-culture of a red organism labeled "Kiel bacillus" that he secured in Berlin about 1890 and which he has carried in his laboratory since that time. This culture is a heavily pigmented strain of the *S. marcescens* type, quite different from the culture received from the Kral collection. Cultures whose labels indicate that they are derived from Breunig's original culture have been obtained from two other laboratories in the United

States. They agree fairly well with the Novy culture and presumably have been derived from his strain. A culture from the Hygienic Institute in Breslau, Germany and one from the American Museum of Natural History Collection are similar to the Lubinsky culture and may have been derived from it.

Under these circumstances, there seems to be good reason for thinking that the Novy culture represents the original Breunig culture and that the Lubinsky culture was wrongly identified. The culture we have called K6 may be identical with some other named culture; but we believe it to be different from Breunig's original culture.

*Serratia* sp. Culture 261 was received April 28, 1924, from the Hygienic Laboratory at Washington, D. C. through the courtesy of Miss Alice Evans. Their stock culture record showed that it was received from the Pasteur Institute, Paris under the label "*Bacillus prodigiosus*" October 1, 1901. It was selected for this study because it was thought to be identical with K6. As noted later, however, there is good reason for thinking that cultures 261 and K6 do not belong to the same species, and certainly neither belongs to *S. marcescens* (*Bacillus prodigiosus*) or to *S. indica*.

#### HISTORICAL

Organisms so closely related to the *Aerobacter aerogenes* group would naturally be expected to be similar in their energy relations. The nitrogen metabolism of the members of the genus *Serratia* is known to be very complex. They readily liquefy gelatin, and break up the protein molecule with liberation of ammonia and certain amines. Very little is known in regard to the action of these organisms on sugars. What little has been done seems to be in relation to gas production.

The production of gas by these organisms has been questioned by various workers and asserted by others. Liborius (1886) found that *Bacillus prodigiosus* grew anaerobically on peptone with the production of gas. Schottelius (1887) stated that this organism had the power of converting sugars into carbon dioxide as well as alcohol. Frankel (1891) accepted this work and

embodied it in his textbook. Scheurlin (1896) disagreed with these workers and stated that the gas produced was formed by the chemical action of succinic acid upon the  $\text{Na}_2\text{CO}_3$  used to neutralize the media. Ritter (1900) confirmed Scheurlin's statement, but later Hefferan (1903) in an extended study of these organisms in a medium free of  $\text{Na}_2\text{CO}_3$  found that in her strain I, a gas bubble appeared in the Smith tube and continually increased in size until about a third of the tube was filled with gas. All of this was absorbed by  $\text{NaOH}$  indicating the absence of gases other than  $\text{CO}_2$ . In studying other members of this group Hefferan found that some of them produced no gas and others produced gases other than  $\text{CO}_2$ .

Scheurlin carried out an extensive investigation in which he showed that ammonia, methyl amine and trimethyl amine could be produced on potato. He further showed that a volatile and a non-volatile acid were produced and identified them as formic and succinic acids.

Bal (1926) in studying the gas produced from soil suspensions found that the carbon dioxide produced by *Bacillus prodigiosus* was not equivalent to the sugar fermented. He also obtained positive tests for alcohol, acetone, and butyric acid, but negative tests for formic and acetic acids. He believed that some other acid may have been produced but could not prove the presence of glycocholic, lactic, oxalic or succinic acids.

#### METHODS

*Media.* The medium used in most of this work was a 1.0 per cent peptone broth containing 3.0 per cent of glucose. An excess of sterilized calcium carbonate was added to the medium after inoculation except in the tubes used for determination of the gas ratio in which case normal sodium hydroxide was used for neutralizing. In estimating the quantity of carbon dioxide produced, the medium was not neutralized. Since carbon dioxide is produced from peptone as well as from glucose a special synthetic medium containing 5 grams  $\text{K}_2\text{HPO}_4$ , 1 gram  $\text{MgSO}_4$ , 5 grams  $\text{NH}_4\text{NO}_3$ , 0.1 gram  $\text{CaCl}_2$  and 5 grams of glucose per liter of water was used for estimating the amount of carbon

dioxide produced from glucose. Before this medium was used, the slight precipitate that formed was filtered off.

### QUALITATIVE METHODS AND RESULTS

Before starting a quantitative analysis of the fermentation products a rather complete preliminary qualitative analysis was made. These tests were made on the media after growth had taken place for four weeks at 25°C.

A 300-cc. aliquot from each flask was extracted with ether for two days in the Kutscher-Steudel (1903) extractor. The extracts

TABLE 1  
*Distilling constants of the volatile acids obtained by Duclaux method*

CULTURE NUMBER	FRACTIONS									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
1377	6.8	13.7	21.1	29.0	37.7	46.9	56.8	68.0	82.2	100.0
Hy	7.1	14.7	22.6	31.0	39.7	49.8	58.3	69.4	82.7	100.0
K5	7.0	14.0	21.5	29.5	37.9	46.8	57.0	68.5	82.4	100.0
Cu37	7.1	14.1	21.5	29.2	37.7	46.8	56.9	68.4	82.1	100.0
K6	7.3	14.9	22.8	31.7	40.9	50.7	60.8	71.6	84.2	100.0
261	6.9	14.1	21.9	30.3	38.9	47.8	57.6	69.0	82.4	100.0
Duclaux's constant for formic.....	5.9	12.2	19.0	26.4	34.4	43.2	52.8	64.6	79.6	100.0
Duclaux's constant for acetic.....	7.4	15.2	23.4	32.0	40.9	50.5	60.9	71.9	84.4	100.0
Authors' constant for acetic.....	7.7	15.7	24.2	33.0	41.9	51.6	61.8	72.6	85.3	100.0

were steam distilled and the distillate tested for the volatile acids. Positive reactions for formic acid were obtained in all cases by the silver nitrate test. Acetic acid was believed to be present in all of the distillates because of the development of the ethyl acetate odor when portions of the neutralized and concentrated liquors were treated with alcohol and sulphuric acid. Propionic and butyric acids were believed to be absent or present in traces only because of the absence of the stronger odor of these two ethyl esters. Since these tests are not decisive, Duclaux distilling constants were run on the remainder of the distillate. The figures obtained are given in table 1.

The constants found would indicate the absence of appreciable amounts of the higher volatile acids. In order to have a very large percentage of the higher volatile acids we would have to have a very high percentage of formic acid present to obtain these low constants. The quantity of formic acid was apparently not great and therefore one is led to believe that the acid is mainly acetic.

The non-volatile portion was used for succinic and lactic acid tests. This liquid was neutralized with barium hydroxide, evaporated to dryness and taken up with a small quantity of water. Enough alcohol was added to give a 90 per cent alcoholic solution. According to Kunz (1903), Moslinger (1902) and Fred and Peterson (1920a), this is an effective means of separating succinic and lactic acids. The insoluble portion was filtered and washed with 90 per cent alcohol, dissolved in water, and the barium precipitated with sulphuric acid. After filtering off the barium sulphate, the liquid was evaporated until crystals formed. They had the characteristic needle shape of succinic acid and gave a positive Neuberg (1901) test in all cases. The mass of crystals formed from the material from culture 261 was very small and the Neuberg test was faint. On the other hand, culture K6 gave a very strong reaction.

The alcohol soluble portions were treated for lactic acid. Portions were tested by the Uffelmann test (Matthews 1920) as well as the thiophene test (Matthews 1920). Both gave positive results in all cases. The remaining liquid was evaporated to a small volume,  $N/2$  zinc sulphate added, the barium sulphate filtered off, and the filtrate evaporated until crystals of zinc lactate formed. These were filtered off, dried in a dessicator until constant in weight, and the percentage water of crystallization determined. Since the results are in close agreement with more accurate data found later, they will not be given here.

One hundred cubic centimeter aliquots of the medium were neutralized and distilled. The distillates were tested for alcohol by the iodoform test and all were strongly positive.

This test, of course, is not specific for alcohol but will also react positively for acetaldehyde and acetone. Aliquots were

tested for reducing power with Fehling's solution and all gave weakly positive tests. This would indicate the production of an aldehyde or other reducing substance. Tests were made for acetyl methyl carbinol (Voges-Proskauer test) and with the exception of culture K6 all were strongly positive. Since acetyl methyl carbinol is a reducing substance, the reduction of Fehling's solution may have been due to this except in the case of culture K6. The Schiff test was also tried and gave very weak reactions. Gunning's iodoform test (Matthews 1920) which is positive for acetone only was tried and a trace of iodoform crystals formed in all cases.

The qualitative tests described indicated the presence of formic, acetic, lactic and succinic acids, acetyl methyl carbinol or 2,3 butylene glycol and alcohol as well as traces of acetone and acetaldehyde. Further qualitative tests in Smith fermentation tubes and in Eldredge tubes (Eldredge and Rogers, 1914) indicated the production of carbon dioxide by all of the organisms, and of hydrogen by cultures 261 and K6.

#### QUANTITATIVE DETERMINATION OF THE PRODUCTS FORMED

For a quantitative estimation of the fermentation products, 1300 cc. portions of the medium were used for each culture. After incubation at 25°C. for six weeks, the determinations were started.

The amounts of sugar remaining were determined by the titration method of Shaffer and Hartmann (1920-1921), the figures later being corrected for the amount of reduction due to formic acid and acetyl methyl carbinol. In all cases most of the sugar had been utilized, as is shown in table 2. Five hundred cubic centimeter aliquots were used for determination of acids, 500 cc. for acetyl methyl carbinol and 2,3 butylene glycol, and 100 cc. for alcohol.

*Volatile acid.* To each of the first aliquots was added enough sulphuric acid to liberate all of the acid. After filtering, they were extracted for five days with ether in the Kutscher-Steudel extractor. The ether was evaporated and an aliquot was titrated with 0.1 N sodium hydroxide to determine the total acid

(table 2). The remainder was steam distilled until about 1800 cc. of distillate had been collected, this amount of distillation having been found to give the full quantity of volatile acid with the least amount of lactic acid with the apparatus used. An aliquot of this was titrated to determine total volatile acid (table 2).

TABLE 2  
*Total acid and alcohol produced by cultures of the genus Serratia*  
Calculated for 100 cc. of culture

CULTURE NUMBER	WEIGHT OF SUGAR FERMENTED	TOTAL 0.1 N ACID	VOLATILE ACID AS ACETIC	NON-VOLATILE ACID AS LACTIC	ALCOHOL AS ETHYL
	grams	cc.	grams	grams	grams
1377	2.7855	156 0	0.1608	1.1628	0 1320
Hy	2.8000	143 7	0.1380	0.9873	0 1932
K5	2.7620	122.7	0 1170	0 9288	0 1297
CU37	2.7374	113 3	0.1830	0 7452	0 1766
K6	2.7708	256.7	0.3618	1.7676	0 1941
261	2.8000	142.3	0.2166	0.9558	0.2847

TABLE 3  
*Distilling constants of volatile acids obtained by Duclaux method*

CULTURE NUMBER	FRACTIONS									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
1377	7 7	15.7	24 0	32 4	41.1	50.3	60.3	71.3	83.9	100 0
Hy	7.5	15.2	23 2	31.6	40 4	49 6	59.6	70.7	83 4	100.0
K5	7.6	15 6	24.0	32.6	41.5	50.8	60.9	71 9	84.4	100 0
CU37	7 7	15.7	23.4	32.5	41.4	50.9	60.9	71.9	84 3	100.0
K6	7.2	14.7	22 5	30 6	39 3	48 6	58 7	69.8	82.9	100.0
261	7 4	15.1	23.0	31.5	40 5	50.1	60 2	71.7	84 9	100.0
Duclaux's constant for formic.....	5.9	12.2	19.0	26.4	34.4	43 2	52 8	64 6	79 6	100.0
Duclaux's constant for acetic.....	7.4	15.2	23.4	32.0	40.9	50.5	60 9	71 9	84.4	100 0
Authors' constant....	7.7	15.7	24.2	33.0	41.9	51.6	61 8	72 6	85 3	100.0

This was neutralized and evaporated to less than 100 cc. Enough 1.0 N sulphuric acid was added to liberate the acids and the Duclaux constants determined.

The separate fractions were saved and reunited with the undistilled liquid. This was used for determination of the



formic acid by the method of Fincke (1913). The results are given in table 4.

*Non-volatile acid.* The non-volatile acid was neutralized with barium hydroxide and evaporated to dryness. The dry salt was taken up in a small quantity of water and enough alcohol added so that a 90 per cent alcoholic solution was obtained in order to effect a separation of the barium lactate from the barium succinate

TABLE 4  
*Estimation of the formic acid by the method of Fincke*

CULTURE NUMBER	MERCURIOUS CHLORIDE	EQUIVALENT FORMIC ACID	
		gram	per cent
1377	0.0524	0.0051	0.18
Hy	0.0791	0.0077	0.27
K5	0.1069	0.0104	0.37
CU37	0.0526	0.0051	0.18
K6	0.6559	0.0639	2.30
261	0.3724	0.0363	1.29

TABLE 5  
*Estimation of amount of succinic acid produced by cultures of the genus Serratia*

CULTURE NUMBER	BARIUM SULPHATE	EQUIVALENT SUCCINIC ACID	SUCCINIC ACID PER 100 CC.		MELTING POINT OF ACID
	gram	gram	gram	per cent	
1377	0.4052	0.2050	0.0342	1.28	182°C.
Hy	0.4034	0.2041	0.0340	1.21	184°C.
K5	0.3594	0.1818	0.0303	1.10	179°C.
CU37	0.2929	0.1482	0.0247	0.90	180°C.
K6	0.9370	0.4741	0.0790	2.85	184°C.
261	0.0370	0.0187	0.0031	0.11	No crystals
Theory.....					185°C.

according to the Moslinger method described by Fred and Peterson (1920a). A light flocculent precipitate of barium succinate formed. After standing for two days, this was filtered off and washed with 90 per cent alcohol. It was then dissolved in hot water and treated with a slight excess of 1.0 N sulphuric acid. The barium sulphate was determined and the amount of succinic acid calculated from this quantity.

The excess of sulphuric acid was carefully precipitated with barium hydroxide and the barium sulphate filtered off. The solution was evaporated nearly to dryness in order that the succinic acid could crystallize out. The crystals were filtered and dried over sulphuric acid and used for melting point determinations as well as for the Neuberg test. The melting points are given in table 5. The Neuberg test in this case was negative with culture 261 but positive with the others.

The alcohol soluble portion was used for determination of lactic acid. The alcohol was evaporated off, water added and 1.0 N zinc sulphate added slowly until no more barium sulphate

TABLE 6  
*Type of lactic acid produced by cultures of the genus Serratia*

CULTURE NUMBER	ZINC LACTATE USED	WATER OF CRYSTALLIZATION		SPECIFIC ROTATION $[\alpha]_D^{20}$	TYPE OF ACID
	grams	grams	per cent		
1377	3.5256	0.4687	13.29	7.5	Levo
Hy	2.9964	0.3900	13.01	7.8	Levo
K5	2.9056	0.3738	12.87	7.5	Levo
CU37	1.8228	0.2348	12.88	7.3	Levo
K6	9.2430	1.6700	18.06	0.0	Inactive
261	3.3751	0.6137	18.18	0.0	Inactive
Theory for active zinc lactate.....			12.97	$\pm 7.52^*$	
Theory for inactive zinc lactate.....			18.17	0.0	

\* Hoppe-Seyler and Araki, 1895.

formed. The material was boiled with animal charcoal to decolorize, then filtered and washed. The clear liquid, containing the zinc lactate was evaporated until crystals formed. These were filtered off, washed carefully with water and then alcohol, and dried until constant in weight. The water of crystallization was determined by heating at 103°C. for five hours. As shown in table 6 all except cultures 261 and K6 produced active acid. The optical rotation of the salts was determined on a solution of 1.0305 grams of the dry salt in 25 cc. of water. Since the optical rotations of the free acid and the salt are opposite, the free acids produced by cultures CU37, K5, 1377, and Hy were levo-rotatory.

*Alcohol.* Alcohol was determined by distilling over 70 to 80 cc. of the 100 cc. aliquot after it had been made alkaline with sodium hydroxide, treating with dichromate solution to oxidize the alcohol to acetic acid, steam distilling and titrating, the alcohol being calculated from the amount of acid formed. The results are given in table 2. Duclaux constants were determined and since these constants (table 7) agreed fairly well with those for pure acetic acid, the alcohol was believed to be practically all ethyl alcohol.

TABLE 7  
*Distilling constants of volatile acid produced from alcohol*

CULTURE NUMBER	FRACTIONS									
	10 cc.	20 cc.	30 cc.	40 cc.	50 cc.	60 cc.	70 cc.	80 cc.	90 cc.	100 cc.
1377	7.9	16.3	24.6	33.4	42.3	52.0	62.3	73.3	85.6	100.0
Hy	7.8	16.1	24.6	33.5	42.6	52.1	62.4	73.1	85.5	100.0
K5	7.8	16.0	24.4	33.2	42.3	51.9	62.2	72.8	85.5	100.0
CU37	7.8	16.1	24.6	33.3	42.3	51.8	62.0	73.1	85.4	100.0
K6	7.8	15.8	24.1	32.8	41.7	51.3	61.4	72.5	84.9	100.0
261	7.8	16.0	24.4	33.1	42.2	51.8	62.0	72.9	85.3	100.0
Authors' constant for acetic.....	7.7	15.7	24.2	33.0	41.9	51.6	61.8	72.6	85.3	100.0

#### ACETYL METHYL CARBINOL AND 2,3 BUTYLENE GLYCOL

The acetyl methyl carbinol and 2,3 butylene glycol were determined according to the method of Harden and Norris (1911-1912) with a few modifications. Harden and Norris stated that their method gave about two-thirds of the real value because of incomplete oxidation of the carbinol and glycol to diacetyl. Since it seemed that a method of obtaining more complete oxidation of these compounds should give higher values, the treatment with bromine was somewhat modified. The aliquots for this determination were carefully distilled, the last part of the distillation being carried out at reduced pressure. The dry residue was extracted with ether and the extract added to the distillate. A 200 cc. aliquot was treated with bromine and exposed to the light of two 40 watt lamps in order to oxidize

the acetyl methyl carbinol and butylene glycol to diacetyl. As the bromine was completely used up more bromine was added until no further reaction took place. The excess of bromine was then carefully neutralized with sulphurous acid, sodium chloride was added and the diacetyl distilled. Since acetyl methyl carbinol and diacetyl have reducing properties while 2,3 butylene glycol has none, the amount of the latter can be estimated by the difference between the two former compounds. The acetyl methyl carbinol was estimated by determining the reducing power of the unoxidized portion and the total diacetyl determined on the

TABLE 8  
*Estimation of acetyl methyl carbinol and 2, 3 butylene glycol*

CULTURE NUMBER	ACETYL METHYL CARBINOL			DIACETYL			BUTYLENE GLYCOL
	Cu <sub>2</sub> O per 100 gm.	Equivalent acetyl methyl carbinol		Cu <sub>2</sub> O per 100 gm.	Equivalent diacetyl		By dif- ference
		gram	per cent		gram	per cent	per cent
1377	0.1631	0.0453	1.62	0.5099	0.4925	17.68	16.06
Hy	0.2287	0.0633	2.26	0.4162	0.4018	14.35	12.09
K5	0.2137	0.0591	2.14	0.2809	0.2713	9.82	7.68
CU37	0.2804	0.0776	2.83	0.5253	0.5071	18.52	15.69
K6	0.0084	0.0023	0.08	0.0080	0.0077	0.27	0.19
261	0.1658	0.0449	1.60	0.3075	0.2970	10.61	9.01
Control				0.0042			
Diacetyl				0.5234	0.5050		

oxidized portion, using the sugar method of Shaffer and Hartmann (1920-1921).

The end point of the titration is sharp as in the sugar determination, but the final color of the liquid is darker, especially if much diacetyl is present. The ratio between the cuprous oxide and the acetyl methyl carbinol was calculated from the equation:  $\text{CH}_3\text{CHOHCOCH}_3 + \text{CuO} = 2\text{Cu}_2\text{O}$ . This gives a value of 1 to 0.2767. Unfortunately this is not correct since copper oxide is not reduced in molecular relationship. An attempt was made to obtain pure acetyl methyl carbinol but without success, so we must use the ratio obtained as the most nearly comparable to the true values. The problem is further

complicated by the fact that if acetaldehyde is produced it will be present here. The ratio of the cuprous oxide to diacetyl was found by reducing the copper solution with pure diacetyl obtained

TABLE 9  
*Production of carbon dioxide on synthetic and peptone media*

CULTURE NUMBER	MEDIA	CALCULATED ON BASIS OF 100 CC.		
		Sugar utilized	Carbon dioxide	
		gram	gram	per cent
1377	Peptone	0.0866	0.0618	
	Peptone	0.1374	0.1241	
	Synthetic	0.1926	0.0546	28.09
	Synthetic	0.1969	0.0546	27.71
Hy	Peptone	0.0716	0.0515	
	Peptone	0.0606	0.0808	
	Synthetic	0.1894	0.0528	27.93
	Synthetic	0.1861	0.0581	31.20
K5	Peptone	0.1256	0.0684	
	Peptone	0.1554	0.1318	
	Synthetic	0.1711	0.0528	31.44
	Synthetic	0.1852	0.0550	29.69
CU37	Peptone	0.1374	0.1241	
	Peptone	0.0998	0.0750	
	Synthetic	0.2024	0.0603	29.78
	Synthetic	0.2205	0.0581	26.33
K6	Peptone	0.0354	0.0097	
	Peptone	0.0168	0.0075	
	Synthetic	0.1969	0.0405	20.56
	Synthetic	0.1710	0.0405	23.66
261	Peptone	0.0562	0.0612	
	Peptone	0.0422	0.0623	
	Synthetic	0.2032	0.0792	38.98
	Synthetic	0.2021	0.0788	38.96

from the Eastman Kodak Company. A portion of this diacetyl was redistilled and a sample collected at a constant temperature of 87.5°C. This was used in a second set of determinations of the reducing power. These agreed with those already obtained.

TABLE 10  
*Analysis of gas according to the method of Burrell*

CULTURE	DETERMINATION	CARBON DIOXIDE	OXYGEN	HYDROGEN	RESIDUAL	CO <sub>2</sub> H <sub>2</sub>
		cc.	cc.	cc.	cc.	
1377	First	42.4	6.1	19.8	29.5	2.14
	Second	85.1	2.2	12.2	17.2	6.97
	Third	111.8	0.0	16.1	24.2	6.94
	Fourth	141.0	0.0	8.4	5.6	25.18
	Fifth	150.2	0.0	6.8	7.8	22.09
	Sixth	169.8	0.0	5.4	8.2	31.44
	Total	700.3	8.3	68.7	92.5	10.19
Hy	First	45.4	5.8	14.4	21.4	3.15
	Second	133.6	0.0	13.3	19.8	10.04
	Third	157.3	0.0	8.2	12.1	19.18
	Fourth	139.7	0.0	4.3	8.4	32.08
	Fifth	149.5	0.2	1.6	2.3	93.43
	Total	625.5	6.0	41.8	64.0	14.96
K5	First	100.4	1.7	0.0	69.5	∞
	Second	180.0	0.2	0.0	17.2	∞
	Third	192.6	0.0	0.0	7.4	∞
	Total	473.0	1.9	0.0	94.1	
CU37	First	74.0	2.4	0.0	115.6	∞
	Second	159.2	0.6	0.0	34.8	∞
	Third	170.6	0.4	0.0	24.0	∞
	Fourth	181.2	0.0	0.0	14.0	∞
	Fifth	171.6	0.0	0.0	24.4	∞
	Total	756.6	3.4	0.0	212.8	
K6	First	12.0	6.3	42.2	87.8	.29
	Second	30.3	8.2	64.5	81.3	.47
	Third	59.8	0.1	68.2	84.2	.87
	Fourth	83.4	1.7	67.9	59.3	1.23
	Fifth	93.3	1.7	39.5	24.1	2.36
	Sixth	140.7	0.5	25.8	13.0	5.45
	Seventh	146.9	0.4	22.3	9.8	6.58
	Eighth	161.1	0.4	13.1	5.6	12.30
	Ninth	160.9	0.3	9.9	9.2	16.25
	Tenth	122.5	0.2	5.5	9.0	22.27
	Eleventh	137.0	0.1	3.7	6.3	37.02
	Twelfth	163.1	0.0	2.6	4.5	62.73
	Thirteenth	148.4	0.0	2.0	3.8	74.20
	Fourteenth	198.6	0.0	2.1	3.6	94.57
	Fifteenth	204.4	0.0	2.2	3.3	92.92
	Sixteenth	189.4	0.0	2.1	3.4	90.19
	Total	2051.8	19.9	373.5	408.2	5.49

TABLE 10—Continued

CULTURE	DETERMINATION	CARBON DIOXIDE	OXYGEN	HYDROGEN	RESIDUAL	$\frac{\text{CO}_2}{\text{H}_2}$
		cc.	cc.	cc.	cc.	
261	First	45.8	3.4	20.5	79.1	2.23
	Second	42.4	2.8	19.9	81.8	2.13
	Third	64.4	8.3	32.6	84.5	1.98
	Fourth	78.8	1.6	38.6	71.2	2.04
	Fifth	84.2	0.6	35.0	86.2	2.40
	Total	315.6	16.7	146.6	402.8	2.15
261	First	47.2	3.7	12.7	91.6	3.72
	Second	75.4	2.2	16.4	102.0	4.59
	Third	85.0	4.6	19.3	86.0	4.39
	Fourth	118.6	2.6	25.7	53.1	4.60
	Total	326.2	13.1	74.1	332.7	4.40

The ratio as well as the results of the determination are given in table 8. We still feel the results are low and in further work of this nature, the LeMoigne (1920) method will be tried in comparison.

*Carbon dioxide.* Carbon dioxide was determined in Eldredge tubes. A known quantity of medium was used and the carbon dioxide absorbed in barium hydroxide. The excess barium hydroxide was titrated with 0.1 N sulphuric acid and the carbon dioxide calculated by the difference. Any carbon dioxide dissolved in the media was determined in the Van Slyke (1917) blood gas apparatus and added to the amount determined by titration. The amounts of sugar used were determined as before.

This group of organisms produces considerable quantities of carbon dioxide from peptone as well as that produced by respiration and fermentation, therefore the synthetic medium described was used for further determinations. The results as given in table 9, show that considerably less carbon dioxide is produced in the synthetic medium. A greater number of other determinations have been run on peptone media but the results are not recorded since they vary as much or more than those given.

*The carbon dioxide-hydrogen ratio.* The comparative amounts of carbon dioxide and hydrogen were determined in the mine gas

analysis apparatus described by Burrell and Siebert (1926). A 2-liter flask of medium containing brom thymol blue was inoculated with the desired organism and the gas collected over mercury. The gases were analyzed as quickly as the burette was filled. When the medium became acid, enough sterilized 1.0 N sodium hydroxide was added with a sterile pipette to neutralize the acid. Several successive determinations were made until the gas ratio seemed to be uniform or until no more growth took place. The results are given in table 10.

TABLE 11  
Percentage amounts of fermentation end products calculated on the basis  
of the sugar fermented

CULTURE NUMBER	LACTIC ACID	SUCCINIC ACID	ACETIC ACID	FORMIC ACID	ALCOHOL	CARBON DIOXIDE	ACETYL METHYL CARBINOL AND BUTYLENE GLYCOL
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1377	39.8	1.3	5.5	0.2	4.7	28.0	17.7
Hy	33.5	1.2	4.5	0.3	6.9	29.5	14.4
K5	32.0	1.1	3.7	0.4	4.7	30.0	9.8
CU37	25.9	0.9	6.4	0.2	6.4	28.1	18.5
K6	59.5	2.9	9.6	2.3	7.0	22.2	0.3
261	34.1	0.1	5.8	1.3	10.2	39.0	10.6
<i>B. coli</i> *	31.9	5.2	18.8		12.8	18.1	0.0
<i>B. aerogenes</i> *	5.5	2.4	5.1	1.0	17.1	41.1	20.0
<i>B. cloacae</i> †	2.0	2.3	1.7	4.3	16.5	41.7	19.0

\* Harden, A. and Walpole S. G. 1905-06.

† Thompson, J. 1911.

#### DISCUSSION OF RESULTS

The results have shown that the organisms of the *Serratia* group are similar in their fermentative ability. They produce acetic, formic, succinic and lactic acids, ethyl alcohol, carbon dioxide and, with the possible exception of culture K6, acetyl methyl carbinol and 2,3 butylene glycol. Hydrogen is produced by all except the *S. indica* cultures. Traces of acetone and acetaldehyde may also be present. The percentage amounts of these materials calculated on the basis of the amount of sugar fermented are compared with results found by Harden and



Walpole (1905-1906) for *Bacillus coli* and *B. aerogenes* and by Thompson (1911) for *B. cloacae* (table 11).

The two cultures of *S. marcescens* gave comparable results and varied only slightly from those of the two *S. indica* cultures. One should expect variations to some extent in a fermentation as complex as this and we may, therefore, consider the results from these four cultures as being essentially the same except in their ability to produce hydrogen. *S. indica* produces no hydrogen. Keyes and Gillespie (1912) and Rogers, Clark and Davis (1914) attach considerable significance to the kind and proportional amounts of gas produced. This difference is important, especially since it correlates with other characters not studied at this time (Breed and Breed, 1926). It is of further interest in that it was noted that cultures of *S. indica* seldom, if ever, gave a bubble of gas in Smith fermentation tubes. On the other hand, cultures of *S. marcescens* were irregular, normally showing no gas but in some instances developing a bubble in the closed arm. More gas was evolved if the fermentation was carried out at a temperature above 25°C. The apparent absence of gas production by organisms that produce carbon dioxide with only traces or no hydrogen may be explained on the basis of the high solubility of carbon dioxide.

From the results, cultures 261 and K6, although having many characters in common with each other and with the organisms above, show certain differences. Culture 261, for example, produces inactive lactic acid and only a trace, if any, of succinic acid, while culture K6 produces not more than a trace of acetyl methyl carbinol or butylene glycol, and produces a large quantity of all the acids in contrast to the other strains studied. On the other hand it produces a smaller amount of carbon dioxide. The lactic acid in this case is also inactive.

Kluyver and Donker (1914) in arranging a natural grouping of bacteria, place *Micrococcus prodigiosus* with the colon organisms since it produces lactic and formic acids, alcohol and 2,3 butylene glycol. The close relationship of the group to the colon-aerogenes group has been recognized for some time. A comparison of the results with those of Harden and Walpole (1905-1906) and

Thompson (1911) (table 11) shows that *Aerobacter aerogenes* and *A. cloacae* are apparently more closely related to the *Serratia* group than to *Escherichia coli*.

This statement was further confirmed by sending a non-pigmented strain of a member of the genus *Serratia* for identification to Dr. J. C. Welden, who found that it grew abundantly on citric acid and uric acid media. In the light of these characters with its reactions to sugar and gelatin, he believed it closely related to but not identical with *A. cloacae*.

It is, of course, recognized that there are some outstanding differences in the comparative amounts of the end products of these fermentations. Especially is this true of lactic acid, acetyl methyl carbinol, butylene glycol, hydrogen and alcohol. The type of fermentation is essentially the same. Even greater differences than those noted above have been obtained by Harden and Penfold (1912) and Neuberg and Gorr (1926) from the same culture of *E. coli*, by varying the growing conditions. No attempt was made in this investigation to duplicate the conditions of previous workers. The results were found under the condition described and undoubtedly they could be altered by making certain variations in these conditions. In fact, it has been found that cultures which ordinarily are Voges-Proskauer negative can be made to give a positive test under certain conditions. As early as (1901) Pakes and Jollyman showed that *B. prodigiosus* as well as other organisms are capable of breaking down formic acid to carbon dioxide and hydrogen. Recently Paine (1927) has shown a destruction of acetyl methyl carbinol by cultures which he believed to be of the colon-aerogenes group. Fred and Peterson (1920B) found that the amount of acetaldehyde produced could be increased if a fixative was added. Many other similar variations of end products from an organism could be cited, especially in the case of organisms which produce a great variety of end products.

The amounts of acetone and acetaldehyde produced were not determined since qualitative tests indicated that these amounts were very small. Bal (1926) reports finding acetone as an end product of a fermentation by *Bacillus prodigiosus* (Culture

forwarded to the authors' of the present paper and found to be a typical culture of *Serratia marcescens*.) Harden and Norris (1911-1912) have stated that acetaldehyde is a precursor of acetyl methyl carbinol. Neuberg and Reinthurth (1923) showed that yeast could be made to synthesize acetyl methyl carbinol if acetaldehyde were added to the medium. On the other hand Grey (1913) and Fred and Peterson (1920B) have shown that the quantity ordinarily produced is very small.

Traces of other products undoubtedly were produced especially from the peptone, but it is believed that the major end products of the sugar fermentation have been accounted for. We find that a large percentage of the end products are accounted for when calculated on the basis of the sugar utilized; but some of these products may be the result of the action of the organisms on the nitrogenous materials. Dox and Plaisance (1917) make the statement that they believe succinic acid is derived from aspartic acid. If this is the case, and since we know that carbon dioxide is produced from peptone, it seems entirely possible that other acids and alcohols could be produced from similar nitrogenous compounds and amino acids. On the other hand, Orla-Jensen (1909) shows by formula how succinic acid may be derived from lactic acid. The source of a number of the materials is still an open question. It should further be noted in totaling the percentages that two different media were used in the determinations. Since carbon dioxide is produced from peptone, a synthetic medium was used for the determination of carbon dioxide from sugar. An attempt was made to use this same medium for determination of other products, but the amount of growth was not sufficient to produce enough end products for analysis.

In view of these results dealing with certain members of the genus *Serratia* and the conclusions of other workers using various species of the colon-aerogenes type, it seems apparent that the groups are closely related. The end products of the fermentation of glucose by these groups are similar and the various products appear in the fermented materials in approximately the same proportion. These relations are most significant when compared with the liquefying strains of the genus *Aerobacter*.

## SUMMARY

Two cultures of *Serratia marcescens* formed the following products: acetic, formic, succinic and levo rotatory lactic acids, ethyl alcohol, acetyl methyl carbinol, 2,3 butylene glycol, carbon dioxide and a small amount of hydrogen.

Two cultures of *Serratia indica* produced the same products except that no hydrogen was found.

Two other cultures of the genus *Serratia* differed from each other, as well as from the two species named. The first of these cultures (261) produced inactive lactic acid. The other culture (K6) also produced inactive lactic acid but in a greater proportion, and produced not more than a trace of acetyl methyl carbinol or 2,3 butylene glycol.

The cultures of the genus *Serratia* Bizio were found to be related in a general way to the genera *Escherichia* Castellani and Chalmers, *Aerobacter* Beijerinck, and *Proteus* Hauser. They are most closely related to the gelatin liquefying species of the genus *Aerobacter*. In fact non-pigmented strains of *Serratia* might properly be classed in the genus *Aerobacter*.

## REFERENCES

- BAL, D. V. 1926. Studies on carbon dioxide production in soil and solution. *Ann. of Applied Biol.*, **13**, 231-243.
- BREED, R. S., AND BREED, M. E. 1924. The Genus *Serratia* Bizio. *Centr. f. Bakt.*, II Abt., **71**, 435-440.
- BREED, R. S., AND BREED, M. E. 1926. *Serratia indica* Bergey, the red chromogenic bacterium originally isolated by Koch in India. *Jour. Bact.*, **11**, 76-77.
- BREUNIG, J. 1888. *Bacteriologische Untersuchung des Trinkwassers der Stadt Kiel im August und September*. Inaug. Diss. Univ. Kiel., 38 pp.
- BURRELL, G. A., AND SEIBERT, F. M. 1926. Sampling and examination of mine gases and natural gas. U. S. D. C. Bureau of Mines, Bull. 197, 105 pp.
- DOX, A. W., AND PLAISSANCE, G. P. 1917. The occurrence and significance of mannitol in silage. *Iowa Agr. Exp. Sta., Res. Bull.* **42**.
- ELDRIDGE, E. E., AND ROGERS, L. A. 1914. The bacteriology of cheese of the Emmental type. *Centr. f. Bakt.*, II Abt., **40**, 5-21.
- FINCKE, H. 1913. Nachweis und Bestimmung der Ameisensaure. *Biochem. Zeit.*, **51**, 253.
- FRAENKEL, C. 1891. *Textbook of Bacteriology*. 3rd Ed. Trans. and edited by J. H. Linsley, Wm. Wood & Co., New York, 376 pp.

- FRED, E. B., AND PETERSON, W. H. 1920A. The fermentation of xylose by bacteria of the aerogenes, paratyphoid B, and typhoid groups. Jour. Inf. Dis., **27**, 539.
- FRED, E. B., AND PETERSON, W. H. 1920B. The production of acetaldehyde by certain pentose-fermenting bacteria. Jour. Biol. Chem., **44**, 29.
- GREY, E. C. 1913. The production of acetaldehyde during the anaerobic fermentation of glucose by *Bacillus coli communis*. Biochem. Jour., **7**, 359.
- HARDEN, A., AND NORRIS, D. 1911-1912. The bacterial production of acetyl methyl carbinol and 2.3 butylene glycol from various substances. Proc. Roy. Soc. London, Ser. B, **84**, 492.
- HARDEN, A., AND PENFOLD, W. J. 1912. The chemical action on glucose of *B. coli communis* obtained by cultivation in the presence of chloroacetate. Proc. Roy. Soc. London, Ser. B, **85**, 415.
- HARDEN, A., AND WALPOLE, S. G. 1905-1906. Chemical action of *B. lactis aerogenes* (Escherich) on glucose. Production of 2.3 butylene glycol and acetyl methyl carbinol. Proc. Roy. Soc. London, Ser. B, **77**, 399.
- HEFFERAN, M. 1903. A comparative and experimental study of bacilli producing red pigment. Centr. f. Bakt., II Abt., **11**, 311.
- HOPPE-SEYLER, F., AND ARAKI, Tr. 1895. Ueber die Einwirkung der bei Sauerstoffmangel im Harne ausgeschiedenen Milchsäure auf polarisirtes Licht und die Rotationswerthe activer Milchsäuren im Allgemeinen. Zeit. physiol. Chem., **20**, 365.
- KEYES, F. G., AND GILLESPIE, L. J. 1912. A contribution to our knowledge of the gas metabolism of bacteria. Jour. Biol. Chem., **13**, 291.
- KLUYVER, A. J., AND DONKER, H. J. L. 1924. The unity in the chemistry of the fermentative sugar dissimilation processes of microbes. K. Akad. Wetenschappen Amsterdam Proc., **28**, 297.
- KUNZ, R. 1903. Über die Bestimmung der Bernsteinsäure im Weine. Chem. Centr. II, 854.
- KUTSCHER, F., AND STEUDEL, H. 1903. Beschreibung eines Ätherextraktionsapparates. Zeit. f. physiol. Chem., **39**, 473.
- LEMOIGNE, M. 1920. Réaction spécifique du 2.3 butyléneglycol et de l'acétylméthylcarbinol, produits de la fermentation butyléneglycolique. Compt. Rend., **170**, 131.
- LIBORIUS, P. 1886. Beiträge zur Kenntnis des Sauerstoffbedürfniss der Bakterien. Zeit. f. Hygiene, **1**, 115.
- MATTHEWS, A. P. 1920. Physiological Chemistry. 3rd Ed., Wm. Wood and Co., New York.
- MOSLINGER, 1902. Über die Säuren des Weines und den Säurerückgang. Chem. Centr., **1**, 231.
- NEUBERG, C. 1901. Ueber den Nachweis der Bernsteinsäure. Zeit. physiol. Chem., **31**, 574.
- NEUBERG, C., AND GORR, G. 1926. Über die saccharogene Bildung von Milchsäure durch verschiedene Bakterien, die Methylglyoxal dismutieren, und über eine einfache Art der Isolierung von Lactat. Biochem. Zeit., **173**, 476.

- NEUBERG, C., AND REINFURTH, E. 1923. Eine neue Form der Umwandlung des Acetaldehyds durch Gärende Hefe. *Biochem. Zeit.*, **143**, 553.
- ORLA-JENSEN, S. 1909. Die Hauptlinien des Natürlichen Bakterien Systems. *Centr. f. Bakt.*, II Abt., **22**, 305.
- PAKES, W. C. C., AND JOLLYMAN, W. H. 1901. The bacterial decomposition of formic acid into carbon dioxide and hydrogen. *Jour. Chem. Soc.*, **79**, 386.
- PAINE, F. S. 1927. The destruction of acetyl-methyl-carbinol by members of the colon-aerogenes group. *Jour. Bact.*, **13**, 269-274.
- RITTER, G. 1900. Zur Physiologie des *B. prodigiosus*. *Centr. f. Bakt.*, II Abt., **6**, 206.
- ROGERS, L. A., CLARK, W. M., AND DAVIS, B. J. 1914. The colon group of bacteria. *Jour. Inf. Dis.*, **14**, 411.
- SCHEURLIN, E. 1896. Geschichtliche und Experimentelle Studien über den *Prodigiosus*. *Arch. Hygiene*, **26**, 1.
- SCHOTTELIUS, M. 1887. Biologische Untersuchungen über den *Micrococcus prodigiosus*. *Ann. Inst. Past*, **1**, 459.
- SHAFFER, P. A., AND HARTMANN, A. F. 1920-1921. The iodometric determination of copper and its use in sugar analysis. *Jour. Biol. Chem.*, **45**, 365.
- THOMPSON, J. 1911. The chemical action of *Bacillus cloacae* (Jordan) on glucose and mannitol. *Proc. Roy. Soc. London, Ser. B.*, **84**, 500.
- VAN SLYKE, D. D. 1917. A method for the determination of carbon dioxide and carbonates in solution. *Jour. Biol. Chem.*, **30**, 347.
- WELDIN, J. C. 1927. The colon-typhoid group of bacteria and related forms. Relationships and classification. *Iowa State College Jour. Science*, **1**, 121.



# STUDIES ON THE PROTEOLYTIC BACTERIA OF MILK

## III. ACTION OF PROTEOLYTIC BACTERIA OF MILK ON CASEIN AND GELATIN

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In Paper II of this series (1928) the action of 229 cultures of proteolytic milk organisms on milk was discussed and the organisms were roughly grouped according to that action. A study of the action of these same organisms on casein and gelatin has also been undertaken to aid further in their grouping.

The tests and methods used in the present work are the same as those used in the work on milk and are as follows: The bromine test for tryptophane, the Salkowski test for indol-acetic acid, the Thomas test for ammonia, and the formol titration for increase in amino nitrogen. In addition, since the casein and gelatin solutions were clear, the "amino-pH number" was determined as an indication of increase in amino nitrogen. The method for this determination has been described elsewhere by one of the authors (Frazier, 1926).

Berman and Rettger (1918) reported that the bacteria which they used required the presence of simpler and more available nitrogen compounds to start the breaking down of purified casein. Therefore, most of the media tried in this work contained in addition to the casein or gelatin small quantities of simpler nitrogenous compounds, such as beef infusion, peptone, or sterile milk. The basic casein medium was made as follows:

Casein (according to Hammarsten).....	10.0 grams
N/1 NaOH.....	4.8 cc.
K <sub>2</sub> HPO <sub>4</sub> .....	2.5 grams
KCl.....	2.0 grams
Distilled water to 1000 cc.	



This medium was modified by the addition of one or more of the following: peptone (Bacto), 1 gram; double strength beef infusion, 10 cc.; glucose, 0.1 gram; or sterile milk, 10 to 100 cc. As much as 20 grams of casein per liter were tried, and the glucose was varied between none and 3 grams per liter in different experiments.

The gelatin medium contained the following: Bacto gelatin, 10 grams;  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 1.2 grams; KCl, 5 grams; peptone, 0.1 gram; beef infusion, 10 cc., glucose, 0.1 gram; and distilled water to make 1000 cc. In one experiment the glucose, peptone, and beef infusion were omitted. The gelatin agar plate method used is described in another paper (Frazier, 1926).

Because of erratic results with *M. varians*, a casein medium was prepared in which lime water was used instead of sodium hydroxide in order that calcium caseinate would be present instead of sodium caseinate. Except that the formula was trebled, the medium was prepared in the same way as the casein solution used for the casein-agar plates and described in Paper I of this series (1928). First 10.5 grams of casein (according to Hammarsten) are soaked in 100 cc. of water. Then 216 cc. of saturated lime water are added and the mixture is shaken until nearly all the casein is in solution. After the addition of 1.05 grams of potassium citrate the shaking is continued until all the casein has dissolved. Then 10 cc. of double strength beef infusion are added and the solution made up to 500 cc. with distilled water. To this solution are added 15 cc. of a 3 per cent solution of calcium chloride and 15 cc. of a phosphate solution of pH 6.7 (2.5 grams of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  and 2.2 grams of  $\text{KH}_2\text{PO}_4$  in 100 cc. of water). The solution is then made up to 1 liter.

#### DATA

In table 1 is shown the action of the cocci and in table 2 the action of the rods in a casein medium containing 0.01 per cent glucose, 0.1 per cent peptone, and 10 cc. of beef infusion. It will be observed that not all the organisms which give an increase in amino-nitrogen in milk can break down casein in the synthetic

medium used. All cultures of *M. perflavus*, *M. varians*, *M. ureae*, *M. freudenreichii* and *Str. liquefaciens* were non-caseolytic. It was thought that the failure of these organisms to decompose the casein might be due to the use of a faulty medium. Therefore the casein medium was varied in a number of ways—the quantity

TABLE 1  
Action of cocci on casein  
(Incubated ten days at 30°C.)

ORGANISM	NUMBER OF CULTURES	pH	NH <sub>3</sub>	SALKOWSKI TEST	BROMINE TEST	AMINO-PH NUMBER	AMINO-N* (FORMOL)*
Control.....		7.0	—	—	—	8.4	cc.
<i>M. citreus</i> .....	31	7.1	+	+	++	7.1	7.2
<i>M. perflavus</i> .....	11	7.2	Sl.	—	—	8.4	0
<i>M. varians</i> .....	9	7.2	—	—	—	8.4	0
<i>M. casei</i> (yellow).....	7	6.9	+	+	+	7.2	6.5
<i>M. percitreus</i> .....	5	7.2	Sl.	Sl.	+	7.8	2.0
<i>M. luteus</i> .....	5	7.1	+	Sl.	+	7.2	7.7
<i>M. cereus</i> .....	3	7.2	—	—	—	7.4	4.6
<i>M. subflavescens</i> .....	2	7.1	Sl.	+	+	7.2	7.2
<i>M. casei</i> (white).....	28	7.0	Sl.	+	++	7.3	5.4
<i>Staph. albus</i> .....	15	7.1	+	Sl.	+	7.3	6.0
<i>M. freudenreichii</i> .....	9	7.2	—	—	—	8.4	0
<i>M. ureae</i> .....	2	7.0	—	—	—	8.4	0
P 147.....	1	7.2	+	+	+	7.3	4.4
P 204.....	1	7.1	Sl.	+	++	7.2	6.2
P 269.....	1	7.4	+	+	++	6.4	21.2
<i>Str. liquefaciens</i> .....	3	7.1	—	—	—	8.4	0
<i>Str. bovis</i> .....	2	7.0	Sl.	+	+	7.2	6.3

\* Increase in amino-N expressed as cubic centimeters of N/10 NaOH per 100 cc. of medium.

Sl. = slight.

of casein and of glucose was varied or small quantities of sterile milk were added. Experiments were made to determine whether the addition of metals would help in the decomposition of the casein. An increase in sugar seemed to cause caseolysis by *Str. liquefaciens*, but none of these modifications of the medium caused any of the other non-caseolytic organisms to split the

casein. Calcium and magnesium seemed to help to some extent the action of organisms which were able to split casein. It will be shown in a following paper that these organisms which are

TABLE 2  
Action of rods on casein  
(Incubated ten days at 30°C.)

ORGANISM	NUMBER OF CULTURES	pH	NH <sub>3</sub>	SALKOWSKI TEST	BROMINE TEST	INDOL	AMINO-DH NUMBER	AMINO-N (FORMOL)*
							cc.	
Control.....		7.0	—	—	—	—	8.4	0
<i>Flavobacterium synnanthum</i> .....	13	7.0	++	+	++	+	6.5	28.8
<i>Flavobacterium lactis</i> .....	1	7.1	Sl.	+	+	—	7.6	3.6
<i>Flavobacterium tremelloides</i> .....	1	6.9	+	—	+	—	7.2	8.2
P 268.....	1	7.6	+	+	++	—	6.2	34.8
<i>Achromobacter coadunatum</i> .....	7	7.2	Sl.	—	Sl.	+	7.9	3.0
<i>Achromobacter liquefaciens</i> .....	6	7.1	+	Sl.	+	—	7.1	10.6
<i>Achromobacter delictatulum</i> .....	1	8.0	++	+	++	—	6.4	27.4
<i>Proteus vulgaris</i> .....	1	7.2	++	++	+	++	7.0	7.8
<i>Alcaligenes bookeri</i> .....	2	7.7	++	+	++	—	6.3	25.8
P 107 ( <i>Escherichia</i> ).....	1	7.3	—	—	—	—	8.4	0
<i>Serratia ruber</i> .....	2	7.4	—	—	—	—	8.4	0
<i>Serratia indica</i> .....	1	7.6	++	+	++	—	6.4	Red
<i>B. albolactis</i> .....	28	7.4	++	+	++	—	6.3	32.0
<i>B. cereus</i> Strain "A".....	8	7.4	++	+	++	—	6.4	32.5
<i>B. cereus</i> , Strain "B".....	3	7.4	++	+	++	—	6.4	28.8
<i>B. vulgatus</i> .....	6	7.4	++	+	++	—	6.4	28.5
<i>B. subtilis</i> .....	2	7.5	+	+	+	—	6.6	23.4
<i>B. simplex</i> .....	2	7.1	Sl.	Sl.	Sl.	—	7.9	3.0
<i>B. mesentericus</i> .....	1	7.1	—	—	Sl.	—	7.8	3.6
<i>B. cohaerens</i> .....	1	7.0	+	+	+	—	7.3	7.4
<i>B. tumescens</i> .....	1	7.5	++	+	++	—	6.4	26.2
<i>B. megatherium</i> .....	1	7.4	++	+	++	—	6.4	29.4
<i>B. ruminatus</i> .....	1	6.9	—	—	—	—	7.2	6.6
<i>B. macerans</i> .....	1	7.2	—	—	—	—	8.4	0
P 67.....	1	7.5	—	—	—	—	8.4	0
P 285.....	1	7.0	—	—	Sl.	—	7.8	2.0

\* See footnote to table 1.

proteolytic in milk, yet do not split casein in synthetic media, are able to break down lactalbumin.

*M. varians* was caseolytic according to the casein agar plates

and according to the bromine test in milk but did not split casein in the sodium caseinate media. Therefore, the organisms were inoculated into a calcium caseinate medium which was the same as the casein agar medium described in Paper I of this series except that the agar was omitted. This decidedly opalescent medium was cleared up by the *M. varians* cultures, and a slight positive bromine test was obtained, although the medium contained such a small quantity of casein that the increase in amino-nitrogen was almost within the limit of error of measurement. The extreme delicacy of the bromine test for free trypto-

TABLE 3  
*Action of organisms on calcium caseinate medium*

ORGANISM	OPALESCENT OR CLEAR	pH	NH <sub>3</sub>	BRO- MINE TEST	TITRAT- ABLE ACIDITY*	AMINO-N (FORMOL)†
Control.....	Opalescent	7.1	—	—	0	0
<i>M. varians</i> .....	Clear	7.0	—	+	2.4	2.0
<i>M. citreus</i> .....	Clear	7.0	Sl.	+	3.7	3.2
<i>Staph. albus</i> .....	Clear	7.0	—	+	4.6	5.2
<i>M. perflavus</i> .....	Opalescent	7.1	—	—	0	0
<i>M. freudenreichii</i> .....	Opalescent	7.1	—	—	0.7	0
<i>Flavobacterium synxanthum</i> ...	Clear	7.0	+	+++	14.4	34.5
<i>Achromobacter liquefaciens</i> ...	Clear	7.0	+	++	10.4	20.5
<i>B. cereus</i> .....	Clear	7.6	++	++	5.8	31.0
<i>B. albolactis</i> .....	Clear	7.5	++	+++	6.2	33.6

\* Increase in acidity expressed as cc. of N/10 NaOH to neutralize 100 cc. of medium to phenolphthalein.

† See footnote to table 1.

phane as a test for caseolysis is shown by the fact that in this case an increase in formol titration of only 0.03 to 0.07 cc. of N/10 NaOH per 5 cc. of medium was detected in a 1 cc. sample.

When the quantity of casein in the medium was trebled, a measurable increase in amino-nitrogen, as well as a distinct positive bromine test, was obtained with *M. varians*. The action of *M. varians* and of some of the other organisms on this calcium caseinate medium is shown in table 3. Some of the organisms can apparently split calcium caseinate more readily than sodium caseinate, but with most of the organisms tried there was little

difference in caseolysis in the two media. The calcium caseinate medium has the advantage that, because of its opalescence, caseolysis can readily be detected by the appearance of the medium.

In casein media the bromine test for free tryptophane is apparently a clear-cut and reliable test for proteolysis. As is shown in tables 1 and 2 the bromine test is positive in all cases of caseolysis except with the three cultures of *M. cereus*, an organism which has given very erratic results on both milk and casein.

TABLE 4  
*Comparison of casein media containing various amounts of glucose*

ORGANISM	NO GLUCOSE					0.01 PER CENT GLUCOSE					0.1 PER CENT GLUCOSE				
	pH	NH <sub>3</sub>	Salkowski test	Bromine test	Amino-pH number	pH	NH <sub>3</sub>	Salkowski test	Bromine test	Amino-pH number	pH	NH <sub>3</sub>	Salkowski test	Bromine test	Amino-pH number
Control.....	7.3	—	—	—	8.47 0	—	—	—	—	8.47 0	—	—	—	—	8.4
<i>M. citreus</i> ..	7.2	Sl.	+	++	7.27 1	+	+	++	++	7.16 0	—	+	+	+	7.4
<i>M. percuti- reus</i> .....	7.5	+	+	+	7.67 2	Sl.	Sl.	+	+	7.86 2	—	—	—	—	7.7
<i>M. casei</i> .....	7.1	—	+	+	7.37 0	Sl.	+	++	++	7.36 0	—	+	+	+	7.3
<i>Str. liquefa- ciens</i> .....	7.3	—	—	—	8.47 1	—	—	—	—	8.45 9	—	Sl.	+	+	7.8
<i>Achromobac- ter coadu- natum</i> .....	7.5	+	++	++	6.97 2	Sl.	—	Sl.	7.97 3	+	+	++	++	++	6.2
<i>Proteus vul- garis</i> .....	7.4	Sl.	+	Sl.	7.57 2	++	++	+	+	7.07 2	++	++	++	++	6.1
<i>B. albolactis</i> .	7.4	++	++	+++	6.37 4	++	+	++	++	6.37 2	++	+	++	++	5.8
<i>B. cereus</i> ....	7.4	++	++	+++	6.37 4	++	+	++	++	6.47 2	++	+	++	++	5.7

The Salkowski test for indol-acetic acid practically parallels the bromine test but does not seem quite so reliable. The test for ammonia also parallels closely the bromine test in this casein medium, which contains only 0.1 per cent glucose. The amino-pH numbers are shown to correspond roughly to the respective formol titration figures.

In table 4 are shown the results of the action of some of the representative cultures on casein media containing 0, 0.01 and 0.1 per cent of glucose respectively. The presence of fermentable

sugar up to 0.1 per cent does not seem greatly to affect the amount of proteolysis or the bromine or Salkowski test, but does nullify the ammonia test in the case of the acid cocci. In these experiments increasing quantities of glucose seemed to cut down caseolysis to some extent in the case of *M. percitreus* and to

TABLE 5  
*Action of cocci on gelatin*

ORGANISM	IN GELATIN SOLUTION				GELATIN STAB LIQUEFACTION	GELATIN-AGAR PLATE	
	pH	NH <sub>3</sub>	Amino pH number	Amino-N* (formol)		HgCl <sub>2</sub> precipitant†	Tannic acid precipitant†
Control.....	7.2	—	8.4	0.0	—	—	—
<i>M. citreus</i> .....	7.3	Sl.	7.3	5.6	+	+	Precipitate
<i>M. perflavus</i> .....	7.4	—	8.4	0.0	+	+	Precipitate
<i>M. varians</i> .....	7.0	—	8.4	0.0	+ (slow)	+	Precipitate
<i>M. casei</i> (yellow).....	7.2	+	6.4	16.7	— to sl.	+	Precipitate
<i>M. percitreus</i> .....	7.8	Sl.	6.8	15.7	+ (slow)	+	Precipitate
<i>M. luteus</i> .....	7.4	+	6.5	22.2	+	+++	++ Ring
<i>M. cereus</i> .....	7.3	—	6.9	18.0	—	+	Precipitate
<i>M. subflavescens</i> .....	7.2	+	6.7	11.3	+	+++	++ Ring
<i>M. casei</i> (white).....	7.1	Sl.	7.3	5.7	— to sl.	+	Precipitate
<i>Staph. albus</i> .....	7.2	+	6.9	11.1	sl. to +	+	Precipitate
<i>M. freudenreichii</i> .....	7.0	—	8.4	0.0	— or +	+	Precipitate
<i>M. ureae</i> .....	7.3	—	8.4	0.0	—	—	—
P 147.....	7.1	+	6.6	16.4	+	++	++ Ring
P 204.....	7.1	+	6.9	7.2	+	+	Precipitate
<i>Str. liquefaciens</i> .....	6.8	—	7.8	2.0	+	++	Heavy precipitate
<i>Str. bovis</i> .....	7.2	+	6.6	12.1	—	+	—
P 269.....	7.3	—	8.4	0.0	—	—	—

\* See footnote to table 1.

† Plus marks refer to width of clear zone about colony.

increase it in the case of *Str. liquefaciens*. It will be noted that *B. albolactis* and *B. cereus*, which resemble each other very closely except that the former ferments lactose, have practically the same caseolytic action when only small quantities of fermentable sugar are present.

The organisms which are able to decompose casein with an increase in amino-nitrogen fall into two distinct groups: a high

amino-nitrogen group and a low amino-nitrogen group. The "high" group might include all organisms which required 20 cc. or more of N/10 NaOH per 100 cc. of media (see tables 1 and 2)

TABLE 6  
Action of rods on gelatin

ORGANISM	IN GELATIN SOLUTION				GELATIN STAB LIQUEFACTION	GELATIN-AGAR PLATE	
	pH	NH <sub>3</sub>	Amino-pH number	Amino-N* (formal)		HgCl <sub>2</sub> precipitant†	Tannic acid precipitant†
Control.....	7.2	—	8.4	0.0	—	—	—
<i>Flavobacterium synzanthum</i> ....	7.6	+++	5.8	56.5	+	+++	++ Ring
<i>Flavobacterium lactis</i> .....	7.4	+	7.1	4.6	—	+	Precipitate
<i>Flavobacterium tremelloides</i> ....	7.2	+	6.3	17.0	+	+++	+ Ring
P 268 .....	8.2	++	5.8	30.8	+	+++	+ Ring
<i>Achromobacter coadunatum</i> ....	8.1	++	6.4	14.3	+	++	+ Ring
<i>Achromobacter liquefaciens</i> ....	7.3	+	7.0	9.4	+	+	Precipitate
<i>Achromobacter delictatulum</i> ....	8.2	+++	6.0	29.4	+	+++	++ Ring
<i>Proteus vulgaris</i> .....	8.2	++	5.8	38.4	+	+++	++ Ring
<i>Alcaligenes bookeri</i> .....	8.2	++	5.8	49.4	+	+++	++ Ring
P 107 ( <i>Escherichia</i> ).....	7.4	—	8.4	0.0	—	—	—
<i>Serratia ruber</i> .....	7.2	—	8.4	0.0	—	+	Precipitate
<i>Serratia indica</i> .....	8.2	+++	6.0	27.8	+	++	+ Ring
<i>B. albolactis</i> .....	8.1	+++	6.0	35.5	+	+++	++ Ring
<i>B. cereus</i> , Strain "A".....	7.8	++	6.0	32.6	+	+++	++ Ring
<i>B. cereus</i> , Strain "B".....	8.0	+++	6.0	34.6	+	+++	++ Ring
<i>B. vulgatus</i> .....	7.5	+++	6.0	30.0	+	+++	++ Ring
<i>B. subtilis</i> .....	8.2	++	6.5	23.5	+	+++	++ Ring
<i>B. simplex</i> .....	7.3	+	6.7	10.3	+	+++	+ Ring
<i>B. mesentericus</i> .....	7.0	+	6.9	10.6	+	++	+ Ring
<i>B. cohaerens</i> .....	7.6	+	6.6	25.0	+	+++	++ Ring
<i>B. tumescens</i> .....	7.5	++	6.0	27.4	+	+++	++ Ring
<i>B. megatherium</i> .....	7.8	++	5.8	35.8	+	+++	++ Ring
<i>B. ruminatus</i> .....	7.6	+	6.3	16.0	+	+++	+ Ring
<i>B. macerans</i> .....	7.0	—	8.4	0.0	+	+	Precipitate
P 67.....	7.6	—	8.4	0.0	+	+	Precipitate
P 285.....	7.2	Sl.	7.0	5.6	—	+	—

\* See footnote to table 1.

† Plus marks refer to width of clear zone about colony.

and the "low" group those organisms which required 10 cc. or less of NaOH. There were no intermediate organisms among those studied.

The action of the proteolytic organisms on gelatin solution, gelatin stab cultures, and gelatin agar plates is shown in tables 5 and 6. It is evident that the organisms may be subdivided into a number of different groups on the basis of their action on gelatin media. This will be discussed in a later paper on classification. As with the casein, however, the organisms may be divided into a negative group, a low amino-nitrogen group, and a high amino-nitrogen group. It is interesting to note that the lactose-fermenting cocci, *M. perflavus* and *M. freudenreichii*, which do not split casein (table 1), also do not break down gelatin. Evidence will be presented in a following paper to show that these organisms are, however, able to decompose lactalbumin.

#### SUMMARY

The action of 229 cultures of proteolytic bacteria from milk on casein and gelatin was studied. Four species of cocci and four of rods were found to cause no increase in amino-nitrogen in casein or gelatin media. The remaining organisms fell into either high or low amino-nitrogen groups in casein and gelatin solutions.

The bromine test was found to be positive in all cases of caseolysis except with *M. cereus*.

*M. varians* was apparently unable to split sodium caseinate but did break down calcium caseinate. Some of the other organisms seem to split calcium caseinate more readily than sodium caseinate.

Increasing amounts of fermentable sugar up to 0.1 per cent did not seem to affect appreciably the amount of caseolysis by most organisms. With *M. percitreus*, however, the caseolysis seemed to decrease with increasing quantities of glucose. Caseolysis by the acid-forming *Streptococcus liquefaciens* seemed to be aided by more fermentable sugar.

A study of the proteolytic milk organisms on casein and gelatin media is of value in the grouping of these organisms.



## REFERENCES

- BERMAN, N., AND RETTGER, L. F. 1918. Further studies on the utilization of protein and non-protein nitrogen. *Jour. Bact.*, **3**, 367-388.
- FRAZIER, W. C. 1926. A method for the detection of changes in gelatin due to bacteria. *Jour. Infectious Diseases*, **39**, 302-309.
- FRAZIER, W. C., AND RUPP, P. 1928 Studies on the protolytic bacteria of milk. I. A medium for the direct isolation of caseolytic milk bacteria. *Jour. Bact.*, **16**, 57.
- FRAZIER, W. C., AND RUPP, P. 1928 Studies on the proteolytic bacteria of milk. II. Action of proteolytic bacteria of milk on milk. *Jour. Bact.*, **16**, 65.

# THE EFFECT OF SURFACE TENSION UPON THE GROWTH OF THE LACTOBACILLI

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The Gram-positive bacteria comprising the genus *Lactobacillus* have been studied comparatively little as a group. Studies of individual members of the lactobacilli have been reported by various workers, but with the exception of *L. acidophilus* and *L. bulgaricus*, which have been the species most frequently studied, knowledge of the cultural characteristics of this group of bacteria is somewhat limited. This is particularly true concerning the points which distinguish the members of this group from one another. The cultural characteristics of *L. bulgaricus* and *L. acidophilus* have been fairly well established. The characteristics of the various caseii types have become known chiefly through the work of European investigators, especially that of Von Freudenreich. Individual characteristics of other members of the lactobacilli are described in the studies made of individual strains by workers interested only in a particular strain.

It is the purpose of this paper to contribute a point of cultural differentiation of certain members of the lactobacilli, based on their ability to grow in a favorable medium the surface tension of which has been depressed with sodium ricinoleate. Differentiation of bacteria by their ability or inability to grow under reduced surface tension has precedence in the work of Larson and his associates (1921); of Ayers, Rupp and Johnson (1923) on streptococci; and of Albus and Holm (1926).

Fifty-eight strains of lactobacilli were included in this study. The identity of each culture and its source, when this was known, are given in the table. The first 17 cultures listed are considered

to be true *L. bulgaricus*. The identification of these strains has been based principally upon the following points: their inability to grow in practically all the bacteriological media commonly used; their inability to grow at a temperature of 15°C.; and their ability to grow at a temperature of 45°C. These are all characteristics which distinguish the true bulgaricus type from the members of the caseii group. Sherman and Stark (1927), in studying the distribution of the lactobacilli in milk, differentiated between the bulgaricus and acidophilus types and the caseii type by their ability to grow at 45° and 15°C. respectively.

The cultures of *L. acidophilus* were obtained from authoritative sources and include, probably, all the strains reported upon in the literature in this country. The strain of *L. bifidus* was obtained from the American Museum of Natural History and was its strain No. 689. The culture of *L. pentoaceticus* was obtained from Dr. E. B. Fred of the University of Wisconsin. The remaining cultures were classified as belonging to the group of *L. caseii*. They all grew at 15°C., did not grow at 45°C. and, in successive transfers, grew well in media that were unfavorable to the growth of *L. bulgaricus*. The strains of *L. caseii* were isolated from the sources stated in the table and are all considered to be similar to, if not identical with, the caseii  $\alpha$  type of Von Freudenreich. None of the CO<sub>2</sub>-producing caseii forms were included in this study.

The medium used was made up of 1 per cent peptone, 1 per cent yeast, 1 per cent beef extract, and 1 per cent lactose. Five-tenths cubic centimeter of a 5-per-cent alcoholic solution of brom-cresol purple per liter was added as an indicator. The medium was divided into four equal portions, and one portion was tubed for use as a control. To each of the three remaining portions a varying amount of pure sodium ricinoleate was added before the medium was tubed. After sterilization the surface tension was determined by the weight-drop method.

Inoculations were made into the test media from mature milk cultures with a platinum loop, and the tubes were incubated at 38°C. The slightest perceptible change in the color of the medium was accepted as an indication of growth and so recorded.

The effect of reduced surface tension upon the growth of the

TABLE 1

*Growth of lactobacilli in media of lowered surface tensions\**

CULTURE NUMBER	CONTROL	pH 7.1			NAME	SOURCE
		45.6 dynes	42.6 dynes	40.4 dynes		
1	G		G	X	<i>L. bulgaricus</i>	
2	G		G	X	<i>L. bulgaricus</i>	A. M. N. H. 598
3	G	G	X		<i>L. bulgaricus</i>	A. M. N. H. 580
4	G	G	X		<i>L. bulgaricus</i>	
5	G		G	X	<i>L. bulgaricus</i>	Yogurt
6	G		G	X	<i>L. bulgaricus</i>	
7	G		G	X	<i>L. bulgaricus</i>	
8	G		G	X	<i>L. bulgaricus</i>	
9	G	G	X		<i>L. bulgaricus</i>	
10	G	G	X		<i>L. bulgaricus</i>	
11	G	G	X		<i>L. bulgaricus</i>	
12	G	G	X		<i>L. bulgaricus</i>	
13	G		G	X	<i>L. bulgaricus</i>	
14	G	G	X		<i>L. bulgaricus</i>	
15	G	G	X		<i>L. bulgaricus</i>	
16	G	G	X		<i>L. bulgaricus</i>	
17	G	G	X		<i>L. bulgaricus</i>	
18	G			G	<i>L. acidophilus</i>	A. M. N. H. 682
19	G			G	<i>L. acidophilus</i>	
20	G			G	<i>L. acidophilus</i>	
21	G			G	<i>L. acidophilus</i>	
22	G			G	<i>L. acidophilus</i>	
23	G			G	<i>L. acidophilus</i>	
24	G			G	<i>L. acidophilus</i>	
25	G			G	<i>L. acidophilus</i>	
26	G			G	<i>L. acidophilus</i>	
27	G			G	<i>L. acidophilus</i>	
28	G			G	<i>L. acidophilus</i>	
29	G			G	<i>L. acidophilus</i>	
30	G			G	<i>L. acidophilus</i>	
31	G			G	<i>L. acidophilus</i>	
32	G			G	<i>L. acidophilus</i>	
33	G			G	<i>L. bifidus</i>	A. M. N. H. 689
34	G		G		<i>L. casei</i>	Swiss cheese
35	G			G	<i>L. casei</i>	Swiss cheese
36	G		G		<i>L. casei</i>	Swiss cheese
37	G		G	X	<i>L. casei</i>	Swiss cheese
38	G		G	X	<i>L. casei</i>	Swiss cheese
39	G		G	X	<i>L. casei</i>	Swiss cheese
40	G		G	X	<i>L. casei</i>	Swiss cheese

TABLE 1—*Concluded*

CULTURE NUMBER	CONTROL	pH 7.1			NAME	SOURCE
		45.6 dynes	42.6 dynes	40.4 dynes		
41	G			G	<i>L. caseii</i>	Cheddar cheese
42	G			G	<i>L. caseii</i>	Cheddar cheese
43	G			G	<i>L. caseii</i>	Cheddar cheese
44	G			G	<i>L. caseii</i>	Cheddar cheese
45	G			G	<i>L. caseii</i>	Cheddar cheese
46	G			G	<i>L. caseii</i>	Milk
47	G			G	<i>L. caseii</i>	Milk
48	G			G	<i>L. caseii</i>	Milk
49	G			G	<i>L. caseii</i>	Milk
50	G			G	<i>L. caseii</i>	Milk
51	G			G	<i>L. caseii</i>	Milk
52	G			G	<i>L. caseii</i>	Milk
53	G			G	<i>L. caseii</i>	Cow feces
54	G		G	X	<i>L. caseii</i>	Swiss cheese
55	G		G	X	<i>L. caseii</i>	Swiss cheese
56	G		G	X	<i>L. caseii</i>	Silage
57	G			G	<i>L. caseii</i>	Silage
58	G			G	<i>L. pentoaceticus</i>	Silage

\* G = growth. X = no growth. A. M. N. H. = American Museum of Natural History.

58 strains of lactobacilli studied is shown in table 1. More than one-half the strains of *L. bulgaricus* were unable to grow in the medium in which the surface tension was depressed with sodium ricinoleate to 42.6 dynes, and none of the *L. bulgaricus* strains showed evidence of growth at 40.4 dynes after an incubation period of seven days at 38°C. Seven of the strains of *L. bulgaricus* showed slight evidence of growth at the end of seven days in the medium depressed to a surface tension of 42.6 dynes. Of this number two cultures (1 and 13) might be considered borderline cultures. Culture 2 was obtained from the American Museum of Natural History as *B. acidi-lactici*, and cultures 5, 6, 7, and 8 were obtained from European workers and had been classified by them as the  $\epsilon$  type of *L. caseii*. However, certain cultural characteristics of these seven strains justify their classification, for the present at least, as *L. bulgaricus*.

All the strains of *L. acidophilus* grew rapidly at the lowest

surface tension employed, as did also the single strain of *L. bifidus*. Although, as has been previously stated, all the strains of *L. casei* included in this study appear to be of the  $\alpha$  type of Von Freudenreich, they are sharply divided by the surface tensions at which they are able to grow. It is perhaps significant that, of the cultures of *L. casei* observed in this study, the growth of those isolated from Swiss cheese, with the exception of culture 35, was inhibited at a higher surface tension than that of the ones isolated from Cheddar cheese, and from milk, and the single strain isolated from cow feces. Cultures 56 and 57, isolated from silage, previously considered identical because of common characteristics, were separated by the surface tension at which each was able to grow.

The ability of an organism or group of organisms to grow at low surface tension may be of value other than as a test method for differentiation. As suggested by Ayers (1923), there is probably indicated "a fundamental difference in structure or metabolism of the cell." It seems likely also that the natural habitat of many types of bacteria is suggested by the surface tension at which each is able to grow. Our present knowledge concerning the relation between surface tension and bacterial growth is merely suggestive.

While the studies here reported are somewhat confined in scope they are submitted as a contribution to the general knowledge of this interesting group of bacteria, with the hope that they may be of some value in the differentiation of the members of the genus *Lactobacillus*.

#### SUMMARY

There is presented a study of the effect of surface tension upon the growth of certain members of the genus *Lactobacillus* which, it is hoped, may be of value in the differentiation of members of this group of bacteria.

#### REFERENCES

- ALBUS, W. R., AND HOLM, G. E. 1926 The effect of surface tension upon the growth of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*. Jour. Bact., 12, 13.

- AYERS, S. H., RUPP, P., AND JOHNSON, W. T. 1923 The influence of surface tension depressants on the growth of streptococci. *Jour. Infec. Dis.*, **33**, 202.
- LARSON, W. P. 1921 The effect of the surface tension of the culture medium on the growth of bacteria. *Abst. Bact.*, **5**, 2.
- SHERMAN, J. M., AND STARK, C. N. 1927 The quantitative and qualitative distribution of lactobacilli in milk. *Jour. Bact.*, **13**, 60.

# A COMPARATIVE STUDY OF THE ACTION OF SODIUM RICINOLEATE UPON BACTERIA

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This study records the results of a comparison of the cellular susceptibility of different species of streptococci and certain other bacteria exposed to the action of sodium ricinoleate under similar physiological conditions. The following microorganisms were investigated: *Streptococcus viridans* (no. 13), *S. pyogenes* (no. 12), *S. equinus* (no. 17), *S. lacticus* (no. 1B), *S. fecalis* (no. 16), streptococci isolated from cases of measles (no. C205, no. C230, no. C231, no. C232), erysipelas (no. C174, no. C175, no. C176, no. C177), and scarlet fever (no. 14E Dochez N. Y. no. 5, no. 14F Dick 1, no. C203 United States Hygienic Laboratory), pneumococci (types I, II, III, and IV), *Staphylococcus albus*, *B. diphtheriae*, *B. tuberculosis*, *B. paratyphosus* A and B, and *B. dysenteriae* (Flexner and Shiga).

Sodium ricinoleate was chosen as the most suitable soap because it dissolves readily in physiological salt solution.

*Preparation of ricinoleic acid.* One hundred grams of commercial castor oil were mixed with 300 cc. of an 8 per cent solution of potassium hydroxide in alcohol, and allowed to stand at room temperature for four hours and at 4°C. for twenty hours.

The sedimented soaps were separated by decantation and filtration, with the aid of suction through a Buchner funnel cooled with ice water. They were then dissolved in water and further treated separately; the alcoholic filtrate, together with the liquid decanted from the solidified soap, was mixed with an excess of water and extracted with ether to remove unsaponified substances. The clear soap solutions were acidified with an excess of 10 per cent hydrochloric acid; the liberated



fatty acids were extracted with ether, washed out several times with water, freed from ether under diminished pressure, and dried in a vacuum desiccator over sulphuric acid; the oily liquid was mixed with one-quarter volume of absolute alcohol and allowed to stand for several days at  $-7^{\circ}\text{C}$ . The saturated fatty acids, which were found in a larger amount in the fraction derived from the soap insoluble in alcohol during the saponification of the oil, crystallized out. The crystals were removed at  $-7^{\circ}\text{C}$ . by decantation and filtration through a folded filter paper. The filtrate was kept at the low temperature as long as the crystals were formed; finally, it was freed from a part of the alcohol by evaporation under diminished pressure, and further cooled until no sediment was deposited. The oily liquid was neutralized with a 4 per cent solution of sodium hydroxide in alcohol. The solidified soap was filtered off and further treated separately. The filtrate was made alkaline to phenolphthalein and was distilled off under diminished pressure. The soaps were dried in a vacuum desiccator over calcium chloride and extracted with ether. Those soluble and insoluble in ether were further treated separately. The sodium soaps were dissolved in water and precipitated with a 10 per cent solution of barium chloride. The barium salts were filtered off with the aid of suction, washed out with water, suspended in 95 per cent alcohol, and heated to  $70^{\circ}\text{C}$ . until dissolved; the solution was cooled to  $50^{\circ}\text{C}$ . and filtered through a filter paper placed in a double-walled metal funnel kept at a temperature of  $40^{\circ}\text{C}$ . The barium salts which solidified in the filtrate at between  $40^{\circ}$  and  $30^{\circ}\text{C}$ . were separated by decantation and further treated separately. The barium salt which remained in the solution below  $30^{\circ}\text{C}$ . and was precipitated by cooling to  $4^{\circ}\text{C}$ . was filtered off. All fractions of barium salt were further purified separately by the above method with 98 per cent alcohol, and finally with absolute alcohol.

The barium salts were suspended in water, and decomposed with 10 per cent hydrochloric acid; the liberated fatty acid was extracted with ether, washed out with 1 per cent sulphuric acid and with water, and freed from ether by evaporation under diminished pressure. The iodine value of some fractions, determined by Wijs' method, was found to be somewhat lower, and that of others, a little higher than the theoretical value. The fraction of fatty acid with the iodine value of 85 was used for the preparation of sodium salt; for that purpose the ricinoleic acid was mixed with two volumes of absolute alcohol and neutralized with 4 per cent sodium hydroxide in alcohol. The soap was filtered off with the aid of suction and dried in a vacuum desiccator. It was a

white powder; and its solution in an 0.85 per cent solution of pure sodium chloride was perfectly clear. A 2 or a 1 per cent solution was sterilized in the autoclave for ten minutes, and certain quantities of that solution were diluted with a sterile salt solution to make up to the different dilutions used in the experiment. A highly diluted solution, 0.01 per cent, of soap should not be heated as it turns opalescent and, therefore, is physiologically less effective.

Sodium chloride used should be free from calcium and magnesium. Old samples of sodium ricinoleate were found to be less active than freshly prepared ones.

All the bacteria studied were grown in streptococcus toxin broth, i.e., beef-infusion broth (pH 7.6 to 7.8) to which 0.02 per cent of glucose was added.<sup>1</sup> For *B. tuberculosis*, only a special broth was used to which 5 per cent of glycerol was added; a small disk of sterilized cork was placed in each test tube to support the bacterial pellicle.

Investigation of the action of sodium ricinoleate was limited to establishing the highest dilutions of soap which inhibit growth and kill the bacteria. The method was the same as that described in a previous paper (Kozlowski, 1925).

#### DISCUSSION OF RESULTS

The presence of a certain amount of sodium ricinoleate in broth inhibited the growth of some strains of the inoculated bacteria completely, while that of some other microorganisms was only slightly retarded or was not even affected in concentrations of 1:1000. In this respect there is as great variation among the streptococci as among other kinds of bacteria (see table 1).

The figures for the growth-inhibiting dilutions of soap represent only a relative value and show that the bacteria which were incubated in the same kind of broth and at the same temperature will react in the manner described. These figures will be much

<sup>1</sup> The possible presence, in the broth, of substances which might weaken the action of the soap should be considered. Broths prepared by special methods used in this laboratory were compared; as, for instance, broth for pneumococcus, *B. diphtheriae*, and streptococcus. (New York, 1927.)

Broth that gave a rich precipitate in twenty-four hours with sodium ricinoleate in a dilution of 1:1000 was found to be less suitable than broth that, under such conditions, formed only a slight turbidity.

lower if such substances as salts of magnesium, calcium, or serum, which bind soap, are mixed with the broth. One cubic centimeter of physiological salt solution added to the broth was enough to influence the reaction on account of an excess of sodium in the medium. The inhibitory test, however, in which water was used as a soap solvent, showed that, in some cases only the highest inhibitory dilutions of soap were somewhat lower than

TABLE 1  
*The action of sodium ricinoleate on bacteria in broth culture*

BACTERIA	HIGHEST INHIBITORY DILUTION OF SOAP IN BROTH	HIGHEST BACTERICIDAL DILUTION OF SOAP IN BROTH (ACTION AT 35°C. IN 7 HOURS)
<i>Streptococcus viridans</i> .....	1:5000	0
<i>Streptococcus pyogenes</i> ....	1:5000	0
<i>Streptococcus equinus</i> .....	1:10,000	0
<i>Streptococcus lacticus</i> ....	1:5000	0
<i>Streptococcus fecalis</i> .....	0	0
Streptococci (measles):		
C230, C232, C205.....	1:10,000	1:5000
C231.....	1:20,000	1:5000
Streptococci (scarlet fever):		
14E, 14F.....	1:20,000	1:5000
C203.....	1:5000	1:5000
Streptococci (erysipelas)...	1:20,000	1:5000
Pneumococci.....	1:20,000	1:10,000
<i>Staphylococcus albus</i> .....	1:5000	1:2000
<i>B. diphtheriae</i> .....	1:20,000	1:2000
<i>B. tuberculosis</i> .....	1:2000	0
<i>B. dysenteriae</i> .....	0	0
<i>B. paratyphosus</i> .....		
<i>B. coli communis</i> .....		

0 = no action in the dilution 1:1000.

those obtained with the soap-salt solution which seemed, to a certain extent, to affect some strains of streptococci; namely, those isolated from cases of erysipelas, scarlet fever (no. 14F), and measles (no. C231), and *S. pyogenes*.

The figures that express the bactericidal dilutions of soap correspond to the given time and temperature; they may increase considerably if the bacteria are in contact with the soap for a

longer time. In many cases some bacteria, pneumococci and certain strains of streptococci, were killed in four hours instead of seven. Transplants made at different time intervals revealed that the bactericidal effect of sodium ricinoleate upon susceptible bacteria is conditioned by the concentration of active soap solution, the amount of bacteria, and the time and the temperature of exposure. The stronger the concentration of the active soap solution, and the higher the temperature (between 0° and 40°C.) the sooner were the bacteria killed. The specific susceptibility of each kind, species, and strain, is the most important factor since, if the particular strain used is non-susceptible, the other factors in the reaction are nullified. Of the streptococci investigated, the strains isolated from cases of scarlet fever and measles were subjected to special study; a slight difference in the susceptibility of the different strains in both groups was observed in tests repeated six times. A comparison of a large number of strains might possibly lead to a more accurate knowledge of the streptococci which belong in these two groups and which have recently attracted so much interest.

Of the bacteria investigated, pneumococci were found to be the most susceptible to the bactericidal action of sodium ricinoleate, types II and III being much more susceptible than type I; they were dissolved by that soap as readily as by bile or sodium oleate. However, they are not more susceptible to the inhibitory action than some of the streptococci.

Sodium ricinoleate may be used in bacteriological research and in diagnostic work as well. The optimum dilution of sodium ricinoleate for a test of the solubility of pneumococci in a broth culture is about 1:5000; higher concentrations of the soap cause a turbidity in the broth which obscures lysis.

The test can be carried out in the following manner: To a small test tube containing 0.8 cc. of broth culture are added 0.2 cc. of a 0.1 per cent solution of sodium ricinoleate in water. The turbidity of the broth culture disappears gradually and the medium becomes clear in from four to fifteen minutes at room temperature. Lysis was accelerated markedly at a temperature of 37°C. Pneumococci, types II and IV, were dissolved much

sooner than pneumococci of types I and III. Pneumococci grown in the "streptococcus" broth were dissolved sooner than in the "pneumococcus" broth. No streptococci studied were dissolved by sodium ricinoleate.

#### SUMMARY AND CONCLUSIONS

Among the streptococci investigated those isolated from cases of erysipelas, measles, and scarlet fever were the most susceptible to the bactericidal action of sodium ricinoleate; they were killed by this soap in a dilution of 1:5000 in about seven hours or sooner at 35°C.; in a control culture, without soap, they survived for more than one week; their growth was inhibited by this soap in a dilution of 1:20,000 or less; in their cellular susceptibility they approach that of pneumococci which were killed by the same soap, under similar conditions, in a dilution of 1:10,000. Pneumococci are dissolved quite readily by sodium ricinoleate in a dilution of 1:5000; and in this regard they differ from other bacteria investigated.

Of the bacilli, *B. diphtheriae* was found to be quite susceptible to the inhibitory, and much less to the bactericidal, action of sodium ricinoleate; *B. tuberculosis* showed a slight susceptibility, as it was inhibited in its growth by this soap in a dilution of 1:2000; *B. paratyphosus*, *B. dysenteriae*, *B. coli communis*, and *S. fecalis* are quite resistant to the inhibitory and bactericidal action of sodium ricinoleate. They are probably adapted to the action of soaps in their natural conditions of growth.

An intermediate place between susceptible and non-susceptible bacteria is taken by some streptococci which are more or less saprophytic, i.e., *S. lacticus*, *S. pyogenes*, and *S. viridans*; they were inhibited in their growth by sodium ricinoleate in a dilution of 1:5000 or lower, but were not killed in a dilution of 1:1000.

The bactericidal effect of sodium ricinoleate is conditioned by the specific cellular susceptibility of the bacteria, and further by the concentration of the soap solution, by the time and the temperature of exposure.

The difference in the cellular susceptibility of various strains of the groups of streptococci isolated from cases of measles, scarlet

fever, and other infections, might be helpful in bacteriological investigations. Sodium ricinoleate may be used instead of bile for dissolving pneumococci in diagnostic routine work.

In conclusion, the author wishes to express his gratitude to Dr. Wadsworth, the director, and to thank Mr. J. Quigley for his kind assistance.

#### REFERENCES

- KOZŁOWSKI, A. 1925 Comparative studies of the action on the pneumococcus of bile acids and unsaturated fatty acids, found in bile in the form of soaps. *Jour. Exper. Med.*, **42**, 453.
- New York, 1927 Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, 86, 87, 88. Baltimore, Williams & Wilkins Co.



# LIMITING FACTORS IN THE LACTIC FERMENTATION

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In the normal course of the lactic fermentation the bacterial growth passes through four stages: the lag period, the period of rapid or logarithmic growth, the period in which there is little or no increase in numbers, and the final period in which fermentation ceases and the cells slowly die.

The changes from the first to the second and from the second to the third period are comparatively rapid and are accompanied by morphological and physiological changes in the cells. The transition from the third to the fourth period is gradual.

In the third period the cells may be in a quiescent condition in which there is little or no multiplication, or the multiplication and death rate may be so nearly balanced that the number counted remains constant. Counts made by the plate method would be likely to give the impression that the latter is the case since only the living cells are counted. If direct microscopic counts are made and the population remains constant it must be assumed either that the cells are quiescent or that they lose their ability to take stain very soon after death.

In our work on this problem we used a microscopic count in order to avoid as far as possible the error introduced into the plate method by the tendency of the lactic streptococci to grow in pairs and chains.

The very large numbers of cells with which it was necessary to work militated against accurate counts, and it was difficult to obtain a series of counts which would give a reasonably smooth curve. However, the constancy with which uniform counts were repeatedly secured under similar conditions indicated that



the numbers obtained must have been fairly representative of the actual conditions. Thus counts made of milk soon after curdling gave very uniformly 1,000,000,000 to 1,200,000,000 cells per cubic centimeter. Special conditions which induced a much greater population and consequently required a higher dilution gave wider variations in the direct count.

It is difficult to ascertain with any high degree of accuracy the relative number of living and dead cells in a lactic culture. An approximation of the proportion of living cells to total cells may be obtained by the comparison of the smallest quantity of culture giving growth in milk with the total count obtained by the direct method. In table 1 are shown the results obtained when direct

TABLE 1  
*The relation of direct count to ability to reproduce in milk*

HOURS FROM INOCULATION	DIRECT COUNT	HIGHEST DILUTION GIVING GROWTH IN MILK
	<i>cells per cubic centimeter</i>	<i>cc.</i>
18	1,500,000,000	$\frac{1}{10,000,000,000}$
20	2,100,000,000	
22	3,900,000,000	
24	3,500,000,000	
42	4,400,000,000	$\frac{1}{1,000,000,000}$
66	4,000,000,000	$\frac{1}{1,000,000,000}$
80	2,400,000,000	$\frac{1}{10,000,000}$
104	3,200,000,000	$\frac{1}{10,000}$

counts on a milk culture and dilutions into milk were made simultaneously. At 104 hours only a relatively small number of cells were sufficiently active to begin reproduction when transferred to fresh milk. At this time, however, the direct count indicated only a small decrease in the number of cells which stained so that they could be counted.

Similar results were obtained when a milk culture was killed by the addition of chloroform. Four hours after 1 per cent of chloroform had been added the direct count was 1,900,000,000; growth was obtained when  $\frac{1}{100}$  cc. was transferred to fresh milk, but none with  $\frac{1}{1000}$  cc. which showed that nearly all the cells were dead. Fifty hours after the chloroform was added the direct count was 1,500,000,000.

Both of these experiments indicate that the ability to stain is retained by the greater number of the cells of a lactic culture for many hours after the ability to reproduce is lost. If the maintenance of a nearly constant population is due to a balance of the birth and death rates the direct count, since the dead cells stain, should show a continued increase. This does not occur and it seems more probable that the stationary condition of the

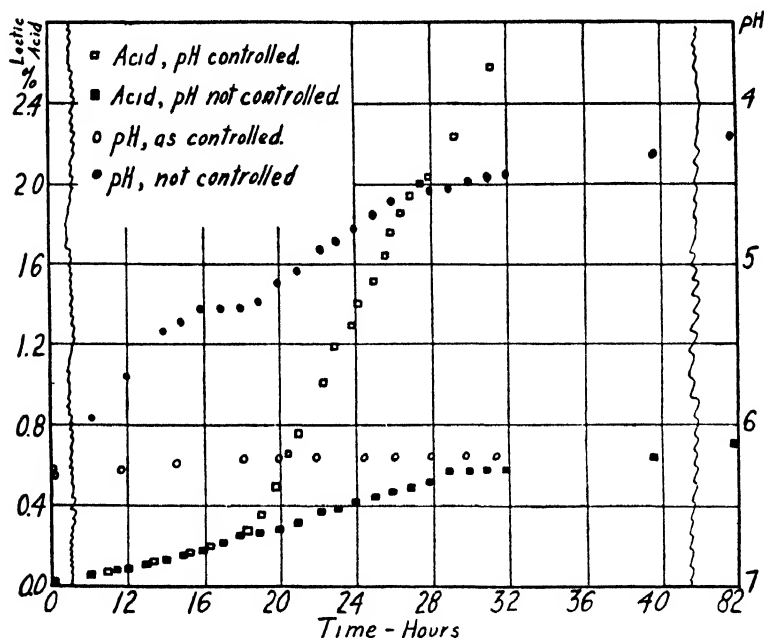


FIG. 1. THE NORMAL RATE OF ACID FORMATION COMPARED WITH THE FERMENTATION IN A MEDIUM HELD AT A CONSTANT HYDROGEN-ION CONCENTRATION

population is due to the fact that the cells go into a quiescent condition in which fermentation may continue but multiplication very nearly ceases.

In lactic cultures this condition occurs when a certain population is reached and this limit is quite constant for definite conditions although it may vary with the culture.

Thus we found that multiplication ceased uniformly at a point

determined by the conditions of the experiment. The population limits of one of the cultures in various media were as follows:

Unbuffered broth.....	60,000,000
Buffered broth.....	480,000,000
Broth with pH held constant.....	1,000,000,000
Milk.....	1,000,000,000
Milk with pH held constant.....	3,600,000,000
Aerated milk with pH held constant.....	5,200,000,000

#### THE EFFECT OF H-ION CONCENTRATION

The fermentation curve follows the population curve through the logarithmic period but if the effect of the acid is removed by neutralization the fermentation curve continues to rise for some time after the increase in population has ceased.

In figure 1 is shown the course of the fermentation under usual conditions and when the reaction was controlled by holding the H-ion concentration at pH 6.2. In the culture with controlled reaction the bacterial population had reached its maximum at about twenty-four hours. The formation of acid continued with only a slight decrease in rate for many hours. The rate of acid formation finally declined and after a time stopped completely. It is evident that if the same factor stops both cell multiplication and acid formation this factor acts on these two cell functions at different concentrations.

Among the causes most frequently suggested for limitation of bacterial growth are the exhaustion of food and the accumulation of the by-products of growth. A number of investigations have shown that under ordinary conditions bacterial multiplication stops while the medium still contains sufficient food to support growth. Some of our observations indicate, however, that with certain lactic cultures the bacterial population and the extent of acid formation in a milk culture are limited by the lack of some particular constituent. This is apparently also true of many of the lactic cultures which do not produce sufficient acid to curdle milk or in which the curdling is materially delayed.

In one culture which was examined multiplication stopped when a population of about 400,000,000 per cubic centimeter was

reached and the milk did not curdle. However, if this culture is grown in milk to which a small amount of pepton has been added the population increases to the normal number and curdling takes place. It is evident that the pepton supplies some essential element for the growth of this culture which was not present in milk in sufficient quantity to promote a normal growth.

This explanation is applicable to certain cultures only and has no direct bearing on the problem of the limits of population in a normal culture.

#### PHYSICAL CROWDING

The constancy with which multiplication stops at a definite number suggests that the physical relation of the cells to one another may have some connection with the limits of growth. The uniform size attained by colonies on solid media also suggests this. A number of investigations have shown that in filtered cultures reinoculated with the same organism the population does not reach the numbers attained in the first propagation (Cornwell and Beer, 1926). This can indicate only that the inhibiting factor is carried in the filtrate and is not directly concerned with the cells themselves. In the investigations on this point we have varied the usual technic by arranging a broth culture so that it could be drawn through a Berkefeld filter and the filtrate returned to the culture flask at frequent intervals. In this way the population of the culture could be reduced below the point at which multiplication usually ceases while the nature of the medium, if it can be assumed that nothing but bacterial cells were removed by the filter, remained unchanged. In these experiments the hydrogen-ion concentration was maintained at a reaction favorable to the growth of *St. lactis*. The results of a typical experiment are shown in figure 2.

The broken line was obtained by calculating, on the basis of the count made at the time filtration began, the number which should have been present at the time each increment of filtered broth was returned to the culture flask. If active multiplication was resumed when the population was reduced the number of bacteria per cubic centimeter should have been greater than the

number calculated from the dilution. This was not found to be the case. The curve for cells counted follows closely that for cells calculated and indicates little or no multiplication.

This experiment is open to the objection that the cells may have passed into the lag phase before filtration was started. However, the culture was held at 30°C. for nearly ten hours after the filtration was started, which should have been sufficient time for reproduction to begin again.

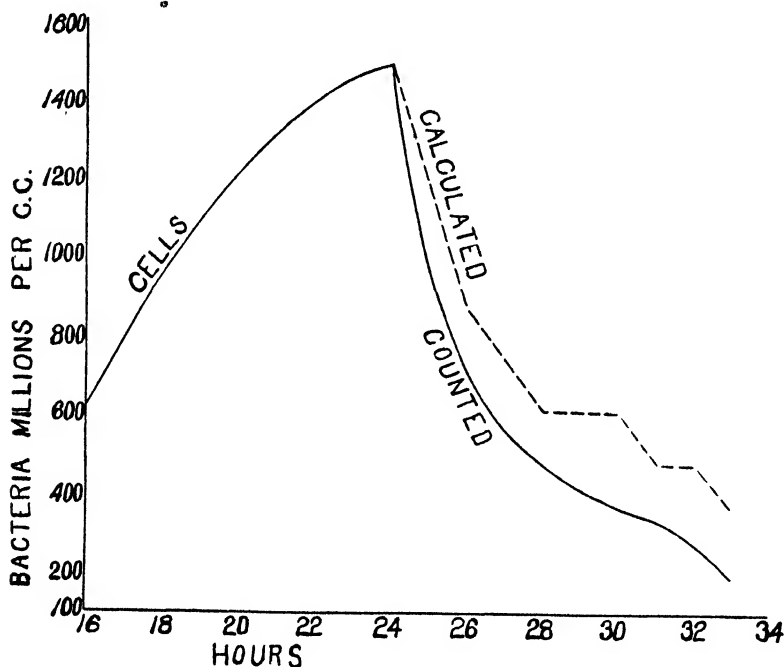


FIG. 2. THE EFFECT OF FILTERING AND RETURNING FILTRATE ON THE BACTERIAL POPULATION OF A BROTH CULTURE

#### THE EFFECT OF PRODUCTS OF METABOLISM

The hydrogen-ion concentration produced by the acid liberated in the fermentation may be considered as a result of metabolism and is usually looked upon as one of the factors, if not the most important factor, in limiting the growth of fermentative bacteria. The limiting hydrogen-ion concentration for cultures of the

*St. lactis* type is usually given as about pH 4.8 (Ayers, Johnson and Davis, 1922). This, however, is the concentration at which logarithmic growth stops and not the final pH. Since the fermentation is continued for some time the hydrogen-ion concentration increases and a milk culture of *St. lactis* allowed to stand for four or five days has a pH of 4.0 or even lower. When the pH is held at a point favorable to continued growth, as for instance pH 5.8 to 6.0, the logarithmic phase is prolonged and the maximum population of the culture is materially increased. Under these conditions multiplication is checked and fermentation finally ceases but at a somewhat higher level than usual.

Under ordinary conditions the hydrogen-ion concentration must be considered a factor in limiting the fermentation, but under the conditions of our experiments in which the hydrogen-ion concentration was controlled another agent was effective.

Obviously acids are not the only inhibiting substances formed. Among the minor products of metabolism may be aldehydes, peroxides, and other compounds having a definite inhibiting effect.

#### THE EFFECT OF AERATION

It was observed that aeration of a culture maintained at a constant hydrogen-ion concentration had the effect of a further prolongation of the fermentation and an increase in the final bacterial population. Thus a culture which has a maximum population of 3,500,000,000 per cubic centimeter in milk held at 30°C. and pH 5.8 to 6.0 has about 5,000,000,000 per cubic centimeter if air is bubbled through the culture. The fermentation is increased accordingly. This may be due to the oxidation or volatilization of inhibitory substances or to an increased oxygen supply.

In an attempt to determine whether volatile substances were driven out of the culture by the air a milk culture was actively aerated with the outlet tube connected with a condenser submerged in a freezing mixture. A small quantity of distillate was collected in this way. This distillate showed no inhibiting effect nor did it change the normal population when added to milk cultures.

These negative results indicate only that no inhibitory substance, condensable in a freezing mixture, was removed by the current of air and do not exclude the possibility that such a substance was formed in the culture or even that it was partly removed by aeration. The possibility that an inhibitory substance was removed by oxidation is eliminated by the fact that as high or even higher counts are obtained when nitrogen is bubbled actively through milk cultures. In fact the highest counts obtained under any conditions were in cultures through which nitrogen was bubbled.

Careful examination of the earlier work in which there was a distinctly larger population in the nitrogen flasks showed that these cultures were not propagated under conditions strictly comparable with those of the aerated cultures. Later experiments under comparable conditions but with another and more active culture showed that while the flasks through which nitrogen was bubbled gave higher counts the difference may not be great enough to be significant. With this particular culture the counts for the unaerated flask were 6,000,000,000 for the aerated flask 7,500,000,000 and for the nitrogen flask 8,000,000,000.

Additional evidence to show that an inhibitory substance is not removed from the culture by the currents of gas was obtained by arranging three culture flasks in series with the nitrogen flowing in order through the first, the second, and the third. It is to be expected that there would be a tendency for volatile substances carried out of the flask to accumulate in the second and third and that this would result in a lower population level in the latter flasks. There was no appreciable difference in the bacterial count or in the fermentation in the three flasks.

The nitrogen experiments prove quite conclusively that the increased growth found in aerated cultures is not due to the oxidation of inhibitory substances or to an increased oxygen supply.

A culture slowly agitated mechanically and one agitated by a stream of nitrogen each changed the reduction potential of the sterilized milk from approximately +0.28 volts to -0.18 volts and maintained this potential at  $-0.20 \pm 0.02$  volts. An aerated culture maintained a somewhat variable but definitely positive

potential, the exact value of which depends apparently on the rate of oxygen supply. It is interesting to note in this connection that when the stream of nitrogen bubbling through a culture was replaced by a stream of air, the potential suddenly rose to a value of the order of  $+0.28$  volts and then decreased somewhat rapidly to a value of the order of  $+0.06$  volts, where it remained reasonably constant as long as the air was passed through. When the air was in turn replaced by nitrogen the potential again changed to the negative range in the vicinity of  $-0.20$  volts. Evidently the adjustment of the processes of the organisms to a sudden supply of oxygen requires a finite amount of time. Details of this adjustment need further investigation.

#### THE ACTION OF UNDISSOCIATED LACTIC ACID

As has been mentioned earlier, hydrogen-ion concentration is usually the factor which causes practical cessation of reproductive and metabolic activities of *St. lactis* cultures. It is true that this factor alone may cause growth and fermentation to cease, as can be demonstrated by the addition of different quantities of a strong acid to a medium (Kolthoff, 1925) into which *St. lactis* is later inoculated. However, it has been shown by Van Dam (1918) and Holwerda (1921) that bacterial activity ceases at lower hydrogen-ion concentrations when the acid added is lactic acid, either free or as a salt. Calculations have shown that the value which is constant under limiting conditions in the presence of added lactates is not hydrogen-ion concentration but the concentration of undissociated lactic acid. Other weak acids may be used to demonstrate the same sort of influence.

Further complications arise, however, for Holwerda (1921) found that the concentration of undissociated lactic acid, which inhibits growth of *St. lactis* in whey, varies with the buffer content of the whey and is affected by the condition of the organisms used for inoculation. In an artificial medium she found that no constant limiting values were obtained.

The conclusions of Van Dam (1918) and Holwerda (1921) have been verified by experiments similar to theirs and by others performed on a somewhat different basis. In the first experiment



skim milk was used. A uniform lot was divided into 225 cc. portions. To each was added a definite quantity of sodium lactate solution which had been adjusted to a hydrogen-ion concentration of pH 6.8. Water was then added to give a total volume of 250 cc. The samples were autoclaved, 50 cc. removed and titrated, and the remainder inoculated with a vigorous culture of *St. lactis*. After 48 hours' incubation at 30°C. determinations were made of titratable acid, hydrogen-ion concentration, and bacterial population. Molality of undissociated lactic acid was calculated from the relationship

$$[\text{H-lactate}] = \frac{[\text{total lactate}]}{1 + \frac{K}{\alpha [\text{H}^+]}}$$

in which brackets indicate mol concentrations, K is the dissociation constant of lactic acid ( $14.7 \times 10^{-5}$ ) and  $\alpha$  is the degree of

TABLE 2  
*Effect of added sodium lactate on the lactic fermentation—skim milk*

INITIAL CONCENTRATION OF LACTATE	LIMITING CONCENTRATION OF LACTATE	LIMITING pH	LIMITING CONCENTRATION H-ION	LIMITING CONCENTRATION (H-LACTATE)	FINAL BACTERIAL COUNT
<i>mols</i>	<i>mols</i>		<i>mols</i> $\times 10^6$	<i>mols</i>	
0.0000	0.0723	4.25	5.62	0.0171	548,068,000
0.0236	0.0958	4.40	3.98	0.0171	948,724,000
0.0473	0.1183	4.48	3.31	0.0181	810,968,000
0.0709	0.1416	4.59	2.57	0.0172	872,064,000
0.0945	0.1625	4.65	2.24	0.0172	660,104,000
0.1181	0.1829	4.74	1.82	0.0165	705,524,000

dissociation of sodium lactate (varying from 0.70 to 0.80 in the range of hydrogen-ion concentration obtained). The results are tabulated in table 2.

The same procedure was followed, whey being substituted for skim milk. The results in table 3 show limiting values approximately one-half as great in the case of whey as when skim milk was used. These agree closely with those obtained by Van Dam (1918).

Similar experiments were carried out in which an artificial

medium was used containing 1.0 per cent pepton, 0.4 per cent beef extract, 0.5 per cent disodium phosphate, and 2.5 per cent glucose. In this medium no constant relations could be found between limitation of growth and fermentation and concentration of undissociated lactic acid.

The effect of lactates was again demonstrated by a different procedure. A culture of *St. lactis* in the medium described in the preceding paragraph was incubated at 30°C. and held at pH 5.8 until counts were practically constant and chemical activity had ceased. The culture was then filtered through Berkefeld filters into sterile flasks. One flask inoculated with *St. lactis* showed no growth. Others were inoculated with a lactate fermenter, *Bact.*

TABLE 3  
*Effect of added sodium lactate on the lactic fermentation—whey*

INITIAL CONCENTRATION OF LACTATE	LIMITING CONCENTRATION OF LACTATE	LIMITING pH	LIMITING CONCENTRATION H-ION	LIMITING CONCENTRATION (H-LACTATE)	FINAL BACTERIAL COUNT
<i>mols</i>	<i>mols</i>		<i>mols</i> × 10 <sup>6</sup>	<i>mols</i>	
0 0000	0 0315	4 30	5 06	0 0069	663,000,000
0 0236	0 0539	4 42	3 80	0.0093	404,000,000
0 0473	0 0760	4 58	2 63	0 0096	304,700,000
0 0709	0 0986	4 69	2 02	0 0099	283,300,000
0 0945	0.1196	4 81	1.55	0 0093	194,000,000
0 1181	0 1407	4 91	1 24	0 0089	241,000,000

*casei* which produced heavy growth. After several days' incubation 3 per cent of glucose was added, the reaction adjusted to pH 6.7, and the cultures again filtered through Berkefeld filters into flasks. Inoculation with *St. lactis* initiated vigorous growth in the filtrates. Briefly stated, decrease of lactate concentration after the attainment of a condition limiting multiplication and growth, enabled the medium to support again the activities of *St. lactis*.

Another type of experiment was carried out in which a culture of *St. lactis* in artificial medium was diluted on successive days with an equal volume of sterile medium of the same composition, except that lactate was present in the same concentration as that in the culture to which the medium was added. It was hoped

that a constant limiting concentration of undissociated lactic acid might be obtained by this procedure, since other influencing factors connected with the bacterial metabolism would be periodically partially diluted out and the effect of the lactate concentration should be preponderant.

A medium of the same composition as that described previously was used. Its initial reaction was pH 6.74. At intervals of twenty-four hours after inoculation 250 cc. of the total 500 cc. were removed for titration and determination of hydrogen-ion concentration. The concentration of accumulated lactate was calculated, and sufficient concentrate of sodium lactate solution was added to 250 cc. of sterile medium to bring its lactate con-

TABLE 4  
*Effect of lactates on the lactic fermentation in artificial medium—diluting out of other factors*

DAY	CONCENTRATION OF LACTATE	pH	H-ION CONCENTRATION	LIMITING CONCENTRATION (H-LACTATE)
	<i>mols</i>		<i>mols</i> $\times 10^5$	<i>mols</i>
1	0 00000	6.74	0 0182	
2	0 00877	6.02	0.0955	(Still active)
3	0.03317	4.21	6.17	0.0087
4	0.04249	4.44	3.63	0 0072
5	0 04895	4.64	2 29	0 0059
6	0.06342	4 67	2 14	0 0069
7	0.06935	4.70	2 00	0 0069

centration to this calculated value. This sterile medium was then added to the remaining 250 cc. of cultured medium. The data and limiting values calculated from the equation previously given are shown in table 4.

It will be seen from the table that the limiting values of hydrogen ion concentration become gradually less as the total lactate concentration increases. Calculation gives values of the same order for successive limiting concentrations of undissociated lactic acid.

In view of widely varying limiting values for concentration of undissociated lactic acid obtained by using Van Dam's technique on artificial medium it must be assumed that the practical

constancy obtained in this experiment is due to the progressive diluting out of still another limiting factor. Either this factor is not found in milk or whey cultures or else, which seems more plausible, its influence is largely neutralized by some component of the milk.

The effect of dilution on cultures in which the organisms had reached the resting phase was tried, the dilutions having been made in several ways. A culture of *St. lactis* in skim milk was held at pH 5.8 and 30°C. until the population had reached its maximum and acid production had fallen to a very low rate. On the assumption that the limiting concentration of undis-

TABLE 5  
*Effects of dilution of various factors on the lactic fermentation*

SERIES NUMBER	SAMPLE NUMBER	CULTURE	STERILE MILK	STERILE WATER	LACTATE 3.827 M	pH AFTER 48 HOURS
		cc.	cc.	cc.	cc.	
I	Check	400				4.74
	1	200		200		4.62
	2	200		172.8	27.2	5.03
	3	200	172.8		27.2	4.93
II	Check	400				4.86
	1	200		200		4.60
	2	200		172.8	27.2	5.11
	3	200	172.8		27.2	4.99

sociated lactic acid had been attained, solutions were prepared, one with sterile milk and sodium lactate solution and another with sterile water and sodium lactate solution in such a way that each contained the quantity of sodium lactate necessary to produce the limiting concentration of undissociated lactic acid at pH 5.8.

Table 5 shows the final hydrogen-ion concentrations obtained in two sets of samples prepared from the culture at an interval of 9 hours. The checks in each case were undiluted: to I, 1 and II, 1 was added sterile water, thereby diluting all factors; to I, 2, and II, 2 was added the sterile water solution of sodium lactate described above, thereby presumably diluting all factors except

the lactate; to I, 3 and II, 3 was added the sterile skim milk solution of sodium lactate previously described, thereby presumably diluting only the factors other than lactate resulting from bacterial metabolism. The fact that the final hydrogen-ion concentration of all the samples including the checks was close to pH 5.0 indicates that metabolism had not entirely ceased when the dilutions were made. It was frequently noted in this work that, after metabolic activity had apparently ceased, it would later be found that acid production was continuing, but at an extremely slow rate. This fact, however, does not vitiate the general conclusions which may be drawn from a comparison of the final hydrogen-ion concentration values.

Dilution of all factors makes it possible for the organisms to carry the hydrogen-ion concentration to higher values than in the undiluted sample, as might be expected.

The data designed to show the effect of diluting all factors except the lactate concentration can not be compared with the data on dilution of all factors because of the fact that the lactate concentration was apparently slightly increased. However, comparison of the results of dilutions I, 2 and II, 2 with I, 3 and II, 3, in all of which the slight artificially produced changes in lactate concentration must have been the same, indicates that milk contains some substance capable of counterbalancing to some extent the influence of some of the self-inhibiting metabolic substances produced by *St. lactis*.

It is admitted that these data do not preclude the possibility that this favorable effect is due to a replenishing of essential food factors which may have been exhausted by the previous bacterial activity.

Experiments were carried out with the artificial medium previously described to determine the effect of *total concentration* of lactates on the *rate of multiplication* of *St. lactis*. Different concentrations of sodium lactate were added to each of two flasks before inoculation, a third flask having no added lactate. Since the initial hydrogen-ion concentration in each flask was about pH 6.8, the corresponding concentration of undissociated lactic acid in each flask must have been less than 0.0001 mol per liter.

Consequently this factor was negligible during the early stages of multiplication. Total lactate concentrations and bacterial populations determined at frequent intervals are plotted in figure 3. No effect of total lactate concentration either on rate of multiplication or on rate of fermentation could be demonstrated.

#### THE FORMATION OF A DEFINITE SUBSTANCE INHIBITING GROWTH

While it is evident that the lactic fermentation is checked, under some circumstances at least, by a certain concentration of

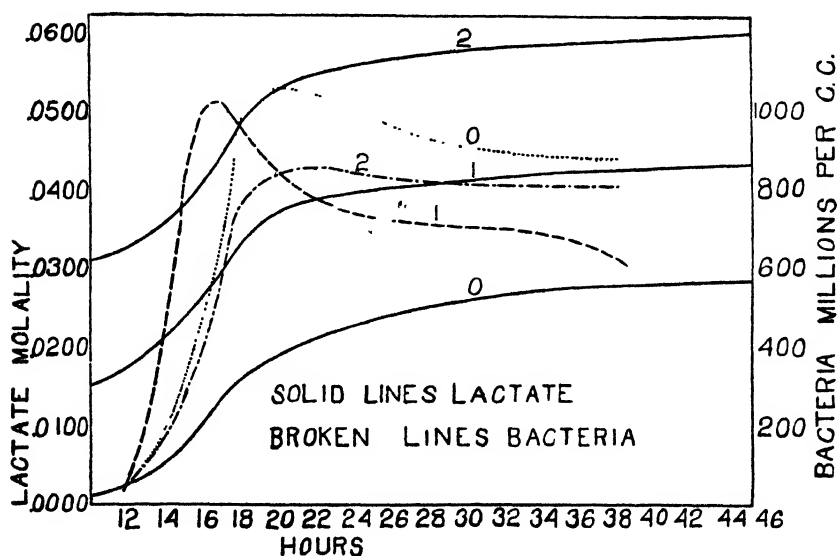


FIG. 3. THE EFFECT OF CONCENTRATION OF LACTATES ON BACTERIAL GROWTH AND ACID FERMENTATION

lactates, this effect is entirely inadequate to explain the cessation of multiplication when a certain population is reached. It may be contended that growth is checked at a concentration of undissociated lactic acid lower than that necessary to stop fermentation, but this explanation would not apply to growth in media in which there is not sufficient carbohydrate to produce a limiting hydrogen-ion or lactic acid concentration. In a medium of this nature it is difficult to avoid the question of the limitation due to a

deficiency of some essential food substance, but it is possible to show that a definite soluble and diffusible inhibiting substance is produced.

The inhibition of the growth of colonies in solid media when the plate is too thickly seeded is a matter of common observation. The inhibition or complete suppression of the colonies of one species by those of another is also frequently observed. Spreading colonies sometimes avoid the colonies of another species, leaving a clear zone around the smaller colony. It is conceivable that this effect may be due to the exhaustion of food, but the diffusion of acid, enzymes, and other soluble products into the surrounding medium is easily demonstrated.

Lactic cultures in sugar agar exhibit an inhibiting effect to a marked degree but this is also true in media containing only traces of fermentable carbohydrates.

The inhibiting action of colonies of lactic bacteria on each other in an agar containing only traces of sugar was demonstrated by pouring a plate with heavy seeding and after it had solidified removing the agar from half of the plate. This empty half was filled with uninoculated agar. The whole layer of agar was covered with a second layer inoculated from the same lactic culture diluted to give well spaced colonies. Half of these colonies were growing over crowded colonies and half were free from any retarding influence except that produced by their own growth. In the upper layer the colonies which were oval or elliptical, averaged 0.14 by 0.22 mm. over the zone of crowded colonies, and 0.33 by 0.45 mm. over the clear zone.

The most plausible explanation of the difference in the size of the colonies is that in the clear zone the size of each colony was limited by the products of its own growth while those colonies which grew over the inoculated half of the first layer were exposed to the products of the congested colonies which diffused into the upper layer and, added to the inhibiting actions of the growth of the colonies themselves, checked their growth before the maximum size was reached.

In a series of simple experiments with collodion sacs it was also demonstrated that an inhibiting substance passes through

the membrane into the surrounding medium. In a typical experiment the sac was filled with a yeast extract broth of twice the usual strength and suspended in a flask of the same medium. The volume of the broth in the flask was about 10 times the volume of that in the sac. The sac was inoculated with a vigorous lactic culture and incubated for two days at 30°C. This flask and a check flask were then inoculated with the same culture and the incubation at 30°C. continued. Beginning at 17 hours after inoculation plate counts were made, and 1 cc. of a yeast broth

TABLE 6  
*Showing the presence of a diffusible inhibiting substance*

TIME FROM INOCULATION	SAC INOCULATED		SAC NOT INOCULATED	
	Concentrated broth added	Bacteria per cubic centimeter	Concentrated broth added	Bacteria per cubic centimeter
<i>hours</i>	<i>cc.</i>		<i>cc.</i>	
17	1	70,500,000	1	308,000,000
18	1		1	
19	1	71,500,000	1	500,000,000
20	1		1	
21	1	92,000,000	1	550,000,000
22	1		1	
24	1	76,500,000	1	550,000,000
25	1		1	
26	1	81,500,000	1	485,000,000
28	1	98,000,000	1	540,000,000
29	1		1	
30	1	94,500,000	1	405,000,000
31	1		1	
32	1	90,000,000	1	380,000,000

made 4 times normal strength was added to each flask at intervals as shown in table 6.

By the addition of the concentrated broth any possible deficiency of essential food material caused by the growth of the culture in the sac was overcome. The addition of the fresh broth caused some increase in population, but a level was reached at which no additional growth resulted from increasing the food supply. Since only 9 cc. of broth were added to about 100 cc. the dilution of by-products from the culture in the sac was not great.



Under these circumstances we may conclude that the marked difference in population in the two flasks could be explained only on the assumption that a definite inhibitory substance was formed in the sac from which it diffused into the surrounding broth. This is in accord with the results obtained by Grundel (1927) who showed that coli cultures had a marked inhibiting action on anthrax bacilli and that this effect was removed when the cultures were shaken with kieselguhr, charcoal, and similar substances. In others words a definite substance was removed by adsorption.

#### DISCUSSION

In considering this problem the question of the multiplication of cells and the conversion of carbohydrates into acid must be separated, although the two phenomena are interdependent.

Fermentation and multiplication proceed hand in hand until some factor having no immediate effect on the fermentation causes the cells to change from a condition of logarithmic growth to one of quiescence. In ordinary media containing fermentable carbohydrates this factor is the attainment of a certain hydrogen-ion concentration which may also check the fermentation at a comparatively low level. If the effect of limiting hydrogen-ion concentration is eliminated the population level rises until a second factor forces the cells into the quiescent state. Under these conditions the fermentation proceeds until it is checked by the concentration of undissociated lactic acid. It is evident that, with other factors eliminated, the population level of a culture is determined by the concentration of a definite, soluble and diffusible substance excreted by the cells. This substance is probably identical with the agent by which one species inhibits another, or is very similar to it, and is the factor which determines the population in a fluid culture or the size of the colony in solid media containing a limited amount of fermentable carbohydrates.

The very definite limit of population under uniform conditions indicates that the production of this hypothetical inhibiting substance is closely concerned in some of the vital processes and is therefore directly proportional to the number of cells. Why it should check growth at different levels under different conditions

is difficult to explain. It is especially difficult to explain the increased population observed when a culture was flooded with an inert gas. However, the combined effect of all unfavorable conditions should be considered rather than the isolated action of a single factor. It is a matter of common observation that a vigorous organism will grow and reproduce itself where a weaker individual will succumb.

#### SUMMARY

Lactic fermentation continues for a time after the bacterial cells cease multiplication, though the same factors appear to limit both.

Control of hydrogen-ion concentration in a culture of *St. lactis* at pH 5.8 to 6.0 permits attainment of greater bacterial populations than when the accumulating acid is not neutralized. Still greater populations may be attained when the culture with controlled pH is agitated with a mechanical stirrer, with air, or with nitrogen, the effectiveness increasing in the order given. Under these conditions the concentration of undissociated lactic acid is the principal factor, though not the only factor, in the limitation of growth and metabolism.

Exhaustion of food factors may limit bacterial activity, but ordinarily the food supply is more than sufficient.

Physical crowding in the mechanical sense is not a factor in the limitation of growth up to limits so far attained.

Volatile products of metabolism, easily oxidizable products of metabolism, total lactate concentration, and self-induced changes in reduction potential have been eliminated as factors in limiting bacterial activity of *St. lactis*.

It has been definitely shown that a substance diffusible through a collodion membrane limits the growth of *St. lactis*.

#### REFERENCES

- AYERS, JOHNSON AND DAVIS 1918 Jour. Infec. Dis., **23**, 290-300.  
AYERS AND MUDGE 1922 Jour. Infec. Dis., **31**, 40-50.  
CORNWELL AND BEER 1926 Ind. Jour. Med. Res., **13**, 789.  
GRUNDEL, M. 1927 Cent. f. Bakt., Abt. I, **104**, 463-473.  
HOLWERDA 1921 Diss. Utrecht.  
KOLTHOFF 1925 Tijdschr. vergelijk. Geneeskunde, **1**, 268.  
VAN DAM 1918 Biochem. Z., **87**, 107.



# STUDIES ON THE PROTEOLYTIC BACTERIA OF MILK

## IV. ACTION OF PROTEOLYTIC MILK BACTERIA ON AMINO ACIDS AND OTHER SIMPLE NITROGENOUS COMPOUNDS

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Although it was the purpose of the present work to group the proteolytic organisms chiefly according to their action on milk and important milk constituents, a study was also made of the use of simple nitrogenous compounds by these organisms in the hope that a better understanding of their nitrogen metabolism might be attained. The organisms were inoculated into synthetic media with various simple nitrogenous compounds as a sole source of nitrogen.

According to Benton (1919) organisms may have individual requirements in amino acid: some may have more catholicity of taste in amino acids than others. Koser and Rettger (1919), however, found that with the organisms they used the various amino acids were similar in their ability to support growth, with the possible exception of tryptophan which was found to be superior to the other amino acids. These workers found that with the exception of the cholera vibrio all the organisms studied could use diammonium phosphate as well as they could use any of the amino acids. Urea was found to be inferior to the amino acids as an immediately available source of nitrogen. Combinations of amino acids or of amino acids and other simple nitrogen-containing compounds were found to be little better than any of the single amino acids.

Gordon and McLeod (1926) worked on the inhibitory action of various amino acids on bacterial growth in peptone broth. They used chiefly delicate pathogenic bacteria, for they found that

*B. coli* and some of the staphylococci were not much influenced. These workers divided the amino acids into three groups: (1) indifferent amino acids, which included arginine, glutamic acid, l-histidine, l-leucine, d-lysine, tyrosine, and valine; (2) favoring amino acids which included taurine, aspartic acid and alanine; and (3) inhibitory amino acids which included: cystine, glycine, phenylalanine and tryptophan. Tryptophan was found to be the most toxic. The amino acids were used in concentrations of from 0.1 to 1 per cent.

Hirsch (1918) discusses the possible courses of decomposition of the various amino acids by microorganisms and records the end-products found by different workers, and Dox (1917) lists possible decomposition products from amino acids due to the action of yeasts and bacteria. References cited by Hirsch and by Koser and Rettger (1919) are not included in the literature reviewed below.

Most reports on the action of organisms on amino acids discuss the action of one or two species of bacteria on only one or two amino acids. Bierema (1909) worked with pure and mixed cultures of soil bacteria and studied their growth on leucine, aspartic acid, tyrosine and asparagine with glycerol or different carbohydrates as sources of carbon. He also tried the following as the sole source of nitrogen: urea, uric acid, hippuric acid, guanidin, formamide, acetamide, and various ammonium salts of inorganic and organic acids. Frouin and Ledebt (1911) showed the effect of monoamino acids on the production of volatile acids by different bacteria. Miyaji (1925) found that his acetic bacteria could decompose l-tyrosine, l-leucine, d-glutamic acid, glycine, dl-phenylalanine and l-histidine. Herzfeld and Klinger (1915) and Sasaki and Otsuka (1921) discussed the decomposition of tryptophan. Raistrick and Clark (1921) studied the action of *B. pyocyaneus*, *B. fluorescens*, *B. prodigiosus* and *B. proteus-vulgaris* on tryptophan and tyrosine. The splitting of tyrosine by *B. proteus-vulgaris* and *B. subtilis* was studied by Tsudji (1917, 1918), by *B. proteus* by Otsuka (1921), and by *B. lactis-aerogenes* by Hirai (1918). The decomposition of tryptophan and of amino-benzoic acid by *B. pyocyaneus* with the

formation of ammonium carbonate was reported by Supiniewski (1924). *B. coli-communis* was used by Hanke and Koessler (1919, 1922) in a study of the decomposition of histidine; and members of the colon-typhoid group were used by Raistrick (1917, 1919). Mayer and Schaeffer (1919) concluded that arginine with its guanidin nucleus and histidine with its imidazole nucleus are essential for the growth of *B. tuberculosis* and that the idea of indispensable amino acids might apply to bacteria as well as to animals. Long (1919) studied the nitrogen metabolism of *B. tuberculosis* in a glycerol-phosphate medium. He found that the organism grew well with urethane, glycine, alanine, asparagine, the acid amides, ammonia, methyl and ethyl amine, or the ammonium salts of the fatty, ketone and hydroxy-acids which correspond to the three amino acids. The splitting of alanine and histidine by *B. tuberculosis* was reported by Campbell (1925). Arai (1921) discussed the decomposition of l-leucine by *B. proteus* and *B. subtilis*. *B. coli*, *B. subtilis* and *B. prodigiosus* are able to split glucosamine according to Takao (1923); and *Bact. tenuis* was found by Abderhalden and Fodor (1913) to be able to split d-glucosamine. Kondo (1923) found that *B. coli* and *Proteus vulgaris* formed hydrogen sulphide and ethyl sulphide from cystine, and that *Proteus vulgaris* also formed mercaptans under certain conditions. Gordon (1924) reported that organisms like those of the colon-typhoid group and the anaerobes, which form hydrogen sulphide, can tolerate large quantities of it. The growth of anaerobes is probably improved by cystine, according to this writer, but delicate organisms do not split cystine and are highly sensitive to it.

In practically all the work reviewed above a source of carbon was provided in the form of a fermentable carbohydrate or similar compound. Thus the nitrogenous compound needed to serve only as a source of nitrogen. The use of asparagine, histidine, and alanine as a sole source of carbon and nitrogen for *B. fluorescens-liquefaciens* was reported by Blanchetière (1916, 1917, 1920).

The fermentation of salts of organic acids with ammonia as the only nitrogen source was used by Ayers, Rupp and Johnson (1919) in their work with the alkali-forming bacteria of milk and by Koser (1923) in work with organisms of the colon-aerogenes group.

## METHODS

In preliminary work the basic medium of Koser and Rettger (1919) was tried. This medium contains distilled ammonia-free water, 1000 cc.; NaCl, 5 grams; MgSO<sub>4</sub>, 0.2 gram; CaCl<sub>2</sub>, 0.1 gram; KH<sub>2</sub>PO<sub>4</sub>, 1 gram; K<sub>2</sub>HPO<sub>4</sub>, 1 gram; and glycerol, 30 grams or glucose, 10 grams. To this medium is added 0.1 per cent of the nitrogenous compound to be tested. It was found that this medium gave rather erratic results and that some organisms were consistently negative in growth although they were positive in the modified medium described below. Sodium chloride in the amount of 0.5 per cent seemed especially inhibitive to some organisms. Therefore only 0.02 per cent of potassium chloride was substituted for sodium chloride in the modified medium; and the quantity of soluble salts was maintained by an increase in the quantity of phosphate buffer salts. The basic medium finally adopted after considerable experimentation contained:

K <sub>2</sub> HPO <sub>4</sub> .....	3.1 grams
KH <sub>2</sub> PO <sub>4</sub> .....	0.8 gram
KCl .....	0.2 gram
MgSO <sub>4</sub> ·7H <sub>2</sub> O .....	0.2 gram
Distilled water to 1000 cc.	

To this basic medium were added glucose or glycerol when desired and the nitrogenous compound in small quantities which varied with the solubility, toxicity, and nitrogen content of the compound. All nitrogen compounds were C. P. Kahlbaum. The pH was adjusted to 7.0 or 7.2. When ammonia was to be the sole source of nitrogen the two phosphate salts in the medium were replaced by 2.5 grams of sodium ammonium phosphate, and 1 per cent of glucose was added. The urea medium contained 1 gram of urea per liter and 0.2 per cent of glucose, and the medium was sterilized by filtration through a Berkefeld filter. All tubes of urea medium were incubated at 30°C. for several days to test for sterility. The amino acids were used with and without sugar. When sugar was added 1 per cent glucose was used. Glycerol was tried but did not satisfy so many organisms nor give so large changes in pH as the glucose. The following amounts of

amino acid were used per liter of medium: sodium aspartate, 3.6 gram; glutamic acid, 3 grams; tyrosine, 1 gram; leucine, 0.5 gram;  $\alpha$  alanine, 3 grams; glycine, 3 grams; and tryptophan, 0.03 gram. Asparagine was used at the rate of 3 grams per liter. In the series of amino acids without sugar a medium with 3 grams of ammonium succinate per liter was used. In preliminary experiments potassium nitrate, potassium nitrite, potassium sulphocyanate, and hippuric acid were also tried as sources of nitrogen but were not found useful.

Light inoculations from fresh agar cultures were used, and incubations were for ten days at 30°C. Then growth, pH, and ammonia were noted. Ammonia was roughly estimated as described in paper II of this series except in the media which originally contained ammonia. The results of these experiments are shown in tables 1, 2 and 3. No growth is indicated in the tables by a minus mark and growth by "slight" or a single plus mark with no further distinction as to the amount of turbidity produced. This was done because it was observed that many of the organisms which barely clouded the medium and might be called negative if turbidity were the criterion for growth were nevertheless fairly active as shown by a marked change in pH or increase in ammonia.

#### DATA

The following organisms did not grow on any of the nitrogenous compounds tried and are therefore not included in the tables: *M. citreus*, *M. casei*, *M. subflavescens*, *Staph. albus*, *Str. liquefaciens*, *Str. bovis*, *Achromobacter liquefaciens* and *Flavobacterium tremelloides*. The cultures of *M. perflavus*, *M. varians*, *M. luteus*, *M. ureae* and *M. freudenreichii* grew only in the urea medium and are found only in table 1.

The action of the organisms on urea is shown in table 1. For the sake of comparison the ability of the same organisms to use ammonia and asparagine as a sole source of nitrogen is indicated in the same table. A number of organisms which can use ammonia and asparagine as nitrogen sources but can not use urea are not included in the table. All cultures of *B. vulgatus*, *B. cereus*, *B.*



*mesentericus*, *B. cohaerens*, *B. megatherium*, and *B. macerans* fall in this category, whereas only one of the cultures called *B. albolactus* breaks down urea. This culture also differs from the other *B. albolactus* cultures in other characteristics. It is interesting to note that with the media used cultures of *M.*

TABLE 1

*Urea, ammonia and asparagine as sole source of nitrogen in a medium with glucose*  
Incubation for ten days at 30°C.

ORGANISM	NUMBER OF CULTURE	NH <sub>3</sub> AS N SOURCE; GROWTH	ASPARAGINE AS N SOURCE; GROWTH	UREA AS N SOURCE		
				Growth	pH	NH <sub>3</sub>
Control .....		—	—	—	7.0	—
<i>M. perflavus</i> .....	11	—	—	+	7.6	++
<i>M. varians</i> .....	9	—	—	+	8.2	++
<i>M. percitreus</i> .....	5	+	+	+	8.0	++
<i>M. luteus</i> .....	5	—	—	+	6.5	—
<i>M. freudenreichii</i> .....	9	—	—	+	8.3	++
<i>M. ureae</i> .....	2	—	—	+	8.4	++
P 147.....	1	+	+	+	8.3	+++
P 269.....	1	+	+	+	7.1	++
<i>Serratia ruber</i> .....	2	+	+	+	8.3	++
<i>Serratia indica</i> .....	1	+	+	+	7.1	Sl.
P 268 ( <i>Serratia</i> ).....	1	+	+	+	8.3	++
<i>Achromobacter coadunatum</i> .....	7	+	+	+	6.0	—
<i>Achromobacter delictatulum</i> .....	1	+	+	+	5.7	Sl.
<i>Proteus vulgaris</i> .....	1	+	+	+	5.9	—
P 107 ( <i>Escherichia</i> ).....	1	+	+	+	8.0	+++
<i>B. subtilis</i> .....	2	+	+	+	7.2	+
<i>B. simplex</i> .....	2	+	+	+	7.8	++
<i>B. tumescens</i> .....	1	+	+	+	7.4	++
<i>B. ruminatus</i> .....	1	+	+	+	8.1	++
P 67 ( <i>Pseudomonas</i> ).....	1	+	+	+	8.2	++

*perflavus*, *M. varians*, *M. luteus*, *M. freudenreichii* and *M. ureae* were able to use urea but not ammonia or asparagine as a source of nitrogen, although the amide group is present in asparagine as well as in urea and ammonia is probably the next step in the degradation of urea.

Most of the organisms which can use urea produce an alkaline

reaction in the medium which is probably due to the liberation of ammonia. It will be observed however that certain organisms like *M. luteus* and *Achromobacter coadunatum* produce no ammonia and cause an acid reaction in the medium. These organisms apparently break down only enough urea to satisfy their nitrogen needs.

The action of the organisms on media which contained glucose and ammonia, asparagine, or one of the amino acids is shown in table 2. Data on the tryptophan medium are omitted from the table because none of the organisms grew to any extent in this medium. The "blank" medium contained no added source of nitrogen. Some few organisms which were apparently able to obtain ammonia from the incubator air (Braun and Goldschmidt, 1927) or enough nitrogen from the light inoculum were able to grow to some extent. This occurred with only four single cultures of organisms. The results in table 2 are in agreement with the conclusions of Koser and Rettger (1919) that if an organism can use ammonia as its only source of nitrogen when a suitable carbon source is furnished, it can also use any of the simpler amino acids as a nitrogen source. As found by Gordon and McLeod (1926), however, certain amino acids may be toxic to some organisms.

A few organisms which were unable to grow in any of the ammonia- or amino-acid media grew to some extent with asparagine as the source of nitrogen. This growth took place in the presence or absence of glucose.

It is apparent that an amino-acid medium which contains a fermentable sugar is not a good differential medium. If, on the other hand, the sugar were omitted and the amino acid were forced to serve as the sole source of both nitrogen and carbon for the organism, some differentiation between organisms might be expected. In table 3 is shown the action of the proteolytic organisms on media in which single amino acids are the only source of both nitrogen and carbon. The results with the amino-acid media without sugar were surprisingly consistent for a simple synthetic medium. Only with cultures of *B. albolactus* were there differences within the species. As will be shown in a fol-

TABLE 2

*Ammonia, asparagine and amino acids as sole source of nitrogen in a medium with glucose*

Incubation for ten days at 30°C.

ORGANISM	NH <sub>4</sub> AS N SOURCE			BLANK NO. N			GLYCINE			GLUTAMIC ACID			TYROSINE			ASPARAGINE			ASPARTIC ACID			ALANINE			LEUCINE		
	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>	Growth	pH	NH <sub>4</sub>			
Control.....	+	7.0	-	-	7.0	-	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-		
<i>M. percitreus</i> .....	+	5.0	7.0	+	5.9	-	+	6.6	-	5.9	-	+	6.6	-	6.4	+	6.8	-	6.4	+	6.8	-	6.4	+	6.8	-	
<i>M. cereus</i> .....	-	7.0	7.0	-	7.0	-	-	7.0	-	7.0	-	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	
P 147.....	+	4.6	5.0	-	4.6	-	+	4.5	-	4.7	-	+	4.5	-	4.5	+	4.5	+	6.8	+	6.8	+	5.8	+	4.6	-	
<i>Flavobacterium synxanthum</i> .....	-	7.0	7.0	-	7.0	-	-	7.0	-	7.0	-	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	
<i>Flavobacterium lactis</i> .....	-	7.0	7.0	-	7.0	-	-	7.0	-	7.0	-	-	7.0	-	7.2	+	7.0	-	7.0	-	7.0	-	7.0	-	7.0	-	
<i>Serratia ruber</i> .....	-	7.0	7.0	-	7.0	-	-	7.0	-	6.5	-	+	4.5	-	6.8	+	6.8	+	8.6	+	8.6	+	6.5	+	4.6	Sl.	
<i>Serratia indica</i> .....	+	4.6	4.6	+	5.1	-	+	5.0	Sl.	5.3	-	+	5.0	Sl.	5.0	+	5.0	+	5.4	-	5.4	-	5.6	+	5.0	-	
<i>Achromobacter coadunatum</i> .....	+	4.6	6.6	-	5.0	Sl.	+	4.5	-	5.9	-	+	4.5	-	6.3	+	6.3	+	7.8	+	7.8	+	6.1	+	4.4	-	
<i>Achromobacter delictatum</i> .....	-	7.0	7.0	-	7.0	-	-	7.0	-	7.0	-	-	7.0	-	7.1	Sl.	7.1	Sl.	7.0	-	7.0	-	7.0	-	7.0	-	
<i>Alcaligenes bookeri</i> .....	+	5.4	7.0	-	5.8	-	+	5.6	-	5.4	-	+	5.6	-	5.7	+	5.7	+	6.0	+	6.0	+	5.6	+	5.1	-	
<i>Proteus vulgaris</i> .....	+	6.3	4.8	-	7.0	-	+	5.1	-	5.0	-	+	5.1	-	6.8	+	6.8	+	7.5	+	7.5	+	6.0	+	4.5	-	
P 107.....	+	*6.0	7.0	+	6.4	-	+	6.6	Sl.	5.5	-	+	6.6	Sl.	6.2	+	6.2	+	5.7	+	5.7	+	6.6	+	6.0	-	
<i>B. albolactis</i> .....	+	5.8	7.0	-	6.7	-	+	6.5	-	5.5	-	+	6.5	-	6.0	+	6.0	+	5.7	-	5.7	-	6.4	+	6.5	-	
<i>B. cereus</i> , Strain "A".....	+	6.5	7.0	+	6.7	-	+	6.6	-	5.5	-	+	6.6	-	6.5	+	6.5	+	5.7	Sl.	5.7	Sl.	6.7	-	7.0	-	
<i>B. cereus</i> , Strain "B".....	+	6.4	7.0	+	6.2	-	+	6.7	-	5.5	-	+	6.7	-	6.0	+	6.0	+	6.1	-	6.1	-	6.7	+	6.8	-	
<i>B. vulgatus</i> .....	+	5.8	7.0	+	5.9	-	+	6.2	-	5.5	-	+	6.2	-	6.4	+	6.4	+	8.2	+	8.2	+	7.4	+	6.7	-	
<i>B. subtilis</i> .....	+	5.8	7.0	+	6.4	-	+	6.2	-	6.2	-	+	6.2	-	6.3	-	6.3	-	6.1	+	6.1	+	5.8	+	6.2	-	
<i>B. simplex</i> .....	+	6.0	7.0	+	6.3	-	+	6.7	-	5.9	-	+	6.7	-	6.3	+	6.3	+	7.3	+	7.3	+	5.8	+	6.8	-	
<i>B. mesentericus</i> .....	+	6.0	7.0	-	6.3	-	+	6.7	-	5.9	-	+	6.7	-	6.3	+	6.3	+	7.3	+	7.3	+	5.8	-	6.8	-	

<i>B. cohaerens</i> .....	+	5.4	-	7.0	-	+	5.2	+	6.7	+	+	6.4	+	+	6.0	+	+	6.1	+	+	+	7.0	-
<i>B. tumescens</i> .....	+	5.8	-	7.0	-	+	5.8	-	±	6.4	-	+	6.8	±	+	6.4	-	+	6.6	-	+	7.0	-
<i>B. megatherium</i> .....	+	5.4	-	7.0	-	+	6.8	-	+	5.4	-	+	6.0	+	+	6.2	-	+	6.3	-	+	7.0	-
<i>B. ruminatus</i> .....	+	5.4	-	7.0	-	+	5.9	Sl.	±	6.4	-	+	6.5	+	+	6.2	+	+	5.8	+	+	5.9	-
<i>B. macerans</i> .....	+	6.0	-	7.0	-	+	6.3	+	+	5.0	-	+	6.4	+	+	7.1	+	+	6.4	Sl.	+	4.8	-
P 67.....	+	4.6	+	5.2	-	+	6.3	+	+	5.0	-	+	6.4	+	+	7.6	+	+	4.7	+	+	4.8	-

\* Of 28 cultures, 21 were positive in medium and 7 negative.

† Not all positive.

TABLE 3  
*Ammonium succinate, asparagine, and amino acids as a sole source of nitrogen and carbon*  
 Incubation for ten days at 30°C.

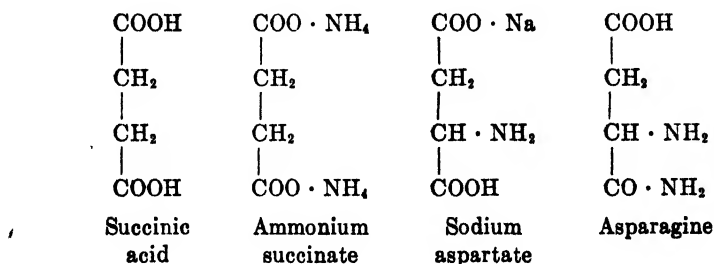
ORGANISM	NUMBER OF ORGANISMS	GROWTH IN AMMONIUM PHOSPHATE WITH GLUCOSE		SODIUM ASPARTATE		ASPARAGINE		GLYCINE		α-ALANINE		GLUTAMIC ACID		TYROSINE*	
		Growth	pH	Growth	pH, NH <sub>4</sub>	Growth	pH, NH <sub>4</sub>	Growth	pH, NH <sub>4</sub>	Growth	pH, NH <sub>4</sub>	Growth	pH, NH <sub>4</sub>	Growth	pH, NH <sub>4</sub>
Control.....		+	7.0	+	7.2	+	7.2	+	7.2	+	7.2	+	7.2	+	7.2
<i>M. percitreus</i> .....	5	+	7.4	+	7.4	+	7.5	+	7.2	+	7.2	+	7.2	+	7.2
<i>M. cereus</i> .....	3	+	7.0	+	7.2	+	7.2	+	7.2	+	7.2	+	7.2	+	7.2
P 147.....	1	+	8.2	+	8.5	+	8.4	+	7.2	+	8.2	+	8.3	+	7.2
P 269.....	1	+	8.1	+	8.5	+	8.3	+	7.2	+	8.0	+	8.5	+	7.2
<i>Flavobacterium synxanthum</i> .....	13	+	7.0	+	7.3	+	7.3	+	7.2	+	7.2	+	7.6	+	7.2
<i>Flavobacterium lactis</i> .....	1	+	7.0	+	7.2	+	7.2	+	7.2	+	7.2	+	7.4	+	7.2
<i>Serratia ruber</i> .....	2	+	7.3	+	7.6	+	7.4	+	7.2	+	7.2	+	7.4	+	7.2
<i>Serratia indica</i> .....	1	+	8.2	+	8.5	+	8.1	+	7.2	+	8.1	+	8.6	+	7.5
P 288 ( <i>Serratia</i> ).....	1	+	8.2	+	8.6	+	8.2	+	7.5	+	8.2	+	8.5	+	7.3
<i>Achromobacter coadunatum</i> .....	7	+	8.0	+	8.5	+	8.1	+	7.2	+	8.2	+	8.2	+	7.2
<i>Achromobacter delictatulum</i> .....	1	+	8.1	+	8.3	+	8.0	+	7.2	+	8.1	+	8.5	+	7.2
<i>Alcaligenes bookeri</i> .....	2	+	7.0	+	7.2	+	7.2	+	7.2	+	7.2	+	7.3	+	7.2
<i>Proteus vulgaris</i> .....	1	+	7.6	+	8.6	+	8.3	+	7.2	+	7.8	+	8.4	+	7.2
P 107.....	1	+	7.6	+	8.4	+	7.8	+	7.2	+	7.9	+	8.4	+	7.2
<i>B. cereus</i> , Strain "A".....	8	+	7.0	+	7.5	+	7.8	+	7.2	+	7.2	+	7.6	+	7.2
<i>B. cereus</i> , Strain "B".....	3	+	7.0	+	7.2	+	7.2	+	7.2	+	7.2	+	7.6	+	7.2
<i>B. vulgatus</i> .....	6	+	7.0	+	7.7	+	7.9	+	7.2	+	7.2	+	7.6	+	7.2
<i>B. subtilis</i> .....	2	+	7.0	+	8.4	+	8.1	+	7.2	+	7.8	+	7.6	+	7.2

<i>B. simplex</i> .....	2	+	-	7.0	+	8.4	+	+	8.2	+	+	7.2	-	+	7.5	+	+	8.0	+	-	-	7.2	-
<i>B. mesentericus</i> .....	1	+	-	7.0	+	7.8	+	+	7.6	+	+	7.2	-	-	7.2	-	-	7.2	-	-	-	7.2	-
<i>B. cohaerens</i> .....	2	+	-	7.0	+	8.2	+	+	8.1	+	+	7.2	-	-	7.2	-	-	7.6	Sl.	-	-	7.2	-
<i>B. tumescens</i> .....	1	+	-	7.0	+	8.2	+	+	7.4	+	+	7.2	-	-	7.2	-	-	7.6	+	-	-	7.2	-
<i>B. megathetium</i> .....	1	+	-	7.0	+	7.5	-	-	7.2	-	-	7.2	-	-	7.2	-	-	7.4	+	-	-	7.2	-
<i>B. ruminatus</i> .....	1	+	-	7.0	+	8.6	+	+	7.2	-	-	7.2	-	-	7.2	-	-	8.2	+	-	-	7.2	-
<i>B. albolactis</i> , "A".....	12	+	-	7.0	+	7.5	+	+	7.7	+	+	7.2	-	-	7.2	-	-	7.7	+	+	-	7.2	-
<i>B. albolactis</i> , "B".....	8	+	-	7.0	+	7.9	+	+	7.2	-	-	7.2	-	-	7.2	-	-	7.9	+	+	-	7.2	-
<i>B. albolactis</i> , "C".....	6	-	-	7.0	+	7.7	+	+	7.2	-	-	7.2	-	-	7.2	-	-	7.2	-	-	-	7.2	-
<i>B. albolactis</i> , "D".....	1	+	-	7.0	-	7.2	-	-	7.2	-	-	7.2	-	-	7.2	-	-	7.5	Sl.	-	-	7.2	-
<i>B. albolactis</i> , "E".....	1	+	-	7.0	-	7.2	-	-	7.2	-	-	7.2	-	-	7.2	-	-	7.2	-	-	-	7.2	-

\* In tyrosine medium, Y = yellow coloration of medium, B = brown, S = salmon color.

lowing paper the cultures which have been grouped together as *B. albolactus*, according to the descriptions of that organism usually given, can be further subdivided on differences of some of their characteristics.

Included in table 3 is an ammonium-succinate medium which is to be compared with the closely related compounds, sodium aspartate and asparagine. These three compounds may all be considered derivatives of succinic acid:



Most of the ammonium succinate was probably present as mono-ammonium succinate after sterilization in the autoclave. Most of the organisms which could use asparagine could also use the aspartate. The only exception was *M. cereus* which produced a rather dubious growth in the asparagine media, both with and without sugar, and should probably be eliminated from tables 2 and 3. *B. megatherium* and 14 cultures of *B. albolactus* were able to use sodium aspartate and did not grow on asparagine. The larger amount of ammonia formed from the asparagine is to be expected because of the amide group. Blanchetière (1917) found that *B. fluorescens-liquefaciens* first attacked the amide group of asparagine and might leave the amino group practically untouched if sugar were present. Long (1919) found the same to be true with *B. tuberculosis*. An examination of table 3 shows that of the organisms which can use ammonia as a nitrogen source in the presence of sugar all the nonspore-forming organisms, both cocci and rods, are able to use ammonium succinate as a sole source of nitrogen and carbon, whereas none of the spore-forming rods are able to do so.

Of the amino acids, other than aspartic acid, the dicarboxylic

glutamic acid served as the best combined source of nitrogen and carbon. All the organisms except *B. mesentericus* and certain *B. albolactus* cultures were able to use it. None of the cultures except P 147 and P 268 grew in the glycine medium and these cultures grew in every medium, including blanks. The  $\alpha$ -alanine medium served as a good differential medium to divide the organisms. None of the organisms appeared to grow to any great extent in the tyrosine medium, although increases in ammonia or changes in the color of the medium were noted with some organisms. In no case was there a marked change in the pH of the tyrosine medium.

The action on these media of the organisms grouped under the name *B. albolactus* deserves special mention. These organisms, which agree with the descriptions of *B. albolactus* usually given, can be split into five distinct subdivisions on the basis of their growth on the amino acids without sugar. Subdivision "A" is positive in sodium aspartate, asparagine and glutamic acid and produces a color change in tyrosine; subdivision "B" is positive in sodium aspartate and glutamic acid and produces a color change in tyrosine; subdivision "C" is positive only in sodium aspartate and colors the tyrosine medium; the organism in subdivision "D" grows only in glutamic acid; and the organism in subdivision "E" grows in none of the media.

It is evident that the organisms may be divided into a number of different groups on the basis of their growth and action on urea, ammonia, asparagine and amino acid media. This will be discussed in more detail in a following paper on classification.

#### SUMMARY

The 229 cultures of proteolytic organisms from milk were inoculated into synthetic media which contained various simple nitrogenous compounds as a sole source of nitrogen, and growth, increase in ammonia and change in pH were noted. The nitrogenous compounds included sodium ammonium phosphate, ammonium succinate, urea, asparagine and the following amino acids: glycine, alanine, leucine, aspartic acid, glutamic acid, tryptophan and tyrosine.



Some of the organisms which can use urea as a sole source of nitrogen cause an alkaline reaction due to the liberation of ammonia, whereas others cause an acid reaction and apparently liberate no free ammonia.

Organisms which can use ammonia as a sole source of nitrogen can apparently use any of the simpler amino acids if the medium contains a fermentable sugar as a source of carbon.

In media which contain no sugar or similar carbon compound and in which the amino acid has to serve as a source of both carbon and nitrogen, results are obtained which may be useful in grouping the organisms.

The proteolytic bacteria can be differentiated into groups on the basis of their growth and action on sugar media which contain ammonia or urea as their only source of nitrogen and on media which contain ammonium succinate, asparagine or various amino acids as a sole source of both carbon and nitrogen.

#### REFERENCES

- ABDERHALDEN, E., AND FODOR, A. 1913 *Z. physiol. Chem.*, **87**, 214-219.  
ARAI, M. 1921 *Biochem. Z.*, **122**, 251-257.  
AYERS, S. H., RUPP, P., AND JOHNSON, W. T. 1919 *Bull.* 782, U. S. Dept. of Agriculture.  
BENTON, A. G. 1919 *Jour. Infec. Dis.*, **25**, 231-247.  
BIEREMA, S. 1909 *Centralbl. f. Bakt.*, II Abt., **23**, 672-726.  
BLANCHETIÈRE, A. 1916 *Compt. rend.*, **163**, 206-209.  
BLANCHETIÈRE, A. 1917 *Ann. Inst. Pasteur*, **31**, 291-312.  
BLANCHETIÈRE, A. 1920 *Bull. soc. chim. biol.*, **2**, 29-36.  
BRAUN, H., AND GOLDSCHMIDT, R. 1927 *Centralbl. f. Bakt.*, I Abt., Orig., **101**, 283-290.  
CAMPBELL, L. K. 1925 *Amer. Rev. Tuberc.*, **11**, 458-470.  
DOX, A. W. 1917 *Proc. Iowa Acad. Sci.*, **24**, 539-545.  
FROUIN, A., AND LEDEBT, S. 1911 *Compt. rend. soc. biol.*, **70**, 24-26.  
GORDON, J. 1924 *Jour. Path. Bact.*, **27**, 123-124.  
GORDON, J., AND McLEOD, J. W. 1926 *Jour. Path. Bact.*, **29**, 13-25.  
HANKE, M. T., AND KOESSLER, K. K. 1922 *Jour. Biol. Chem.*, **50**, 131-191.  
HERZFELD, E., AND KLINGER, R. 1915 *Centralbl. f. Bakt.*, I Abt., **76**, 1-12.  
HIRAI, K. 1918 *Acta Scholae Med.*, Univ. Imperial Kyoto, **2**, 425-432.  
HIRSCH, P. 1918 *Biochemie in Einzeldarstellungen*. IV. Die Einwirkung von Mikroorganismen auf die Eiweisskörper.  
KOESSLER, K. K., AND HANKE, M. T. 1919 *Jour. Biol. Chem.*, **39**, 539-584.  
KONDO, M. 1923 *Biochem. Z.*, **136**, 198-202.  
KOSER, S. A. 1923 *Jour. Bact.*, **8**, 493-520.

- KOSER, S. A., AND RETTGER, L. F. 1919 Jour. Infec. Dis., **24**, 301-321.
- LONG, E. R. 1919 Amer. Rev. Tuberc., **3**, 86-108.
- MAYER, A., AND SCHAEFFER, G. 1919 Compt. rend. soc. biol., **82**, 113-115.
- MIYAJI, K. 1925 Jour. Chem. Soc., Japan, **45**, 391-450.
- OTSUKA, I. 1921 Biochem. Z., **114**, 81-87.
- RAISTRICK, H. 1917. Biochem. Jour., **11**, 71-77.
- RAISTRICK, H. 1919 Biochem. Jour., **13**, 446-458.
- RAISTRICK, H., AND CLARK, A. B. 1921 Biochem. Jour., **15**, 76-82.
- SASAKI, T., AND OTSUKA, I. 1921 Biochem. Z., **121**, 167-170.
- SUPINIEWSKI, J. 1924 Comp. rend. soc. biol., **90**, 1111-1112.
- TAKAO, K. 1923 Z. physiol. Chem., **131**, 307-318.
- TSUDJI, M. 1917 Acta Scholae Med., Univ. Imperial Kyoto, **1**, 439-448. Abstr. in Physiol. Abstracts **2**, 320.
- TSUDJI, M. 1918 Acta Scholae Med., Univ. Imperial Kyoto, **2**, 115-123. Abstr. in Physiol. Abstracts **3**, 165.



# THERMOPHILIC ORGANISMS FOUND IN CULTURE MEDIA

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During the course of a study of the thermophilic organisms associated with milk (Prickett, 1928), it was discovered that batches of certain media were difficult to sterilize. Standard Methods of Milk Analysis (1927), page 10, state that "Sterilization shall be accomplished by heating in the autoclave for twenty minutes after the pressure reaches 15 pounds." Standard Methods of Water Analysis (1925), page 96, state that

All media shall be sterilized in the autoclave at 15 pounds (120°C.) for fifteen minutes after the pressure has reached 15 pounds. . . . Media shall be sterilized in small containers and these must not be packed closely together. No part of the medium shall be more than 2.5 cm. from the outside surface of the glass or from the surface of the medium.

In this study certain batches of media that had been filled into standard bacteriological glass test tubes of 1.5 cm. diameter were loosely packed in baskets and heated in the autoclave for twenty minutes after the pressure had reached 20 pounds (126.7°C.). In this laboratory it is a routine practice, after the air has been driven out of the autoclave, to leave the drain valve slightly open so that a small amount of steam is continually escaping throughout the heating period. These media were apparently sterile as long as they were not incubated above 37°C. If, however, these same lots of media were incubated at 56° or 63°C. in less than twenty-four hours numerous distinct, small colonies ("pin-point" to larger size) appeared, first, just beneath the surface of the slanted area and not in the deep part of the

agar. On continued incubation tiny colonies appeared at lower levels in the agar. Later it was found necessary to heat media of the same type in the autoclave for thirty to thirty-five minutes at 20 pounds pressure to insure sterility.

Isolations made from these "sterile" media showed that a pure culture was often present. Since several lots of media gave the same results it was decided to investigate the ingredients of the media to learn the source of these organisms. Witte's peptone, Bacto-peptone, Bacto-peptonized milk and Bacto-tryptophane broth, ingredients used in preparing these media, gave no indication of the presence of thermophiles. The agar and Liebig's beef extract, other ingredients used in these media, were found to contain numerous viable spore-forming thermophiles. Three lots of agar were used, one of which was ordinary shredded agar, another Bacto-agar, and the third a powdered agar from the American Agar Company.

When the methods of manufacture of these two materials are recalled it is not surprising that they contain large numbers of thermophilic organisms. The temperatures resulting from cooking meat under reduced pressure as in preparing beef-extract would not harm the thermophiles but instead would favor their development, and the spores apparently are able to withstand the desiccation of the concentrated extract. A large proportion of the agar of commerce, prepared from certain algae growing in the sea, is manufactured in Japan. Methods employed by the Japanese in the manufacture of this material are described by Smith (1904). The algae, after air-drying and bleaching in warm weather, are boiled with water and then strained, cooled, cut in strips, and dried. The resulting time and temperature relations obtained in the manufacture offer favorable conditions for the development of thermophiles, not to mention the possibility of inoculating subsequent batches by the continued use of the same equipment.

In examining the culture media ingredients, they were vigorously shaken with sterile water, allowed to stand a few minutes and reshaken, then plated, using nutrient agar known to be sterile, and incubated at 56°C. Representative colonies de-

veloping on the plates were picked. Approximately 25 cultures were secured from "sterile" media and media ingredients. These were recognized as falling into two types apparently identical with *Bacillus aero-thermophilus* Weinzierl and *B. thermophilus-aquatilis-liquefaciens* Michaelis. The cultural characteristics and the nomenclature of these organisms are discussed elsewhere.<sup>1</sup>

H. G. Harding (1927) also noted that certain culture media contained very heat-resistant organisms. Recently this laboratory received supplies from The Digestive Ferments Company which had been processed to remove the heat-resistant forms. These materials have been found to be free from the organisms that will develop on plates incubated at 56° or 63°C. which is in marked contrast with the numerous colonies developing on similar plates prepared from the unprocessed material. Such a processed medium can be sterilized by using the time and temperature recommended by the Standard Methods.

Media have been observed that stood at room temperature for three weeks and were then incubated at 37°C. for one week with no sign of bacterial growth. Yet when the same media were incubated at 56°C. growth of thermophilic organisms appeared in less than twenty-four hours. All of the observations here reported indicate that a bacteriologist can be certain his culture media is actually sterile only when he incubates representative portions of it at 56°C. as well as at 25° and 37°C.

#### REFERENCES

- HARDING, H. G. 1927 Thermophilic organisms in milk and their significance. Doctor's Thesis, University of Wisconsin, Madison, Wis.
- PRICKETT, P. S. 1928 Thermophilic organisms in milk. Jour. Bact., 15, 28.
- SMITH, HUGH M. 1904 The seaweed industries of Japan. Bull. Bureau of Fisheries, 24, 135-165.
- Standard Methods of Milk Analysis. 1927 American Public Health Association. New York. 5th ed., 1927, 55 pp.
- Standard Method of Water Analysis. 1925 American Public Health Association. New York. 6th ed., 1925, 119 pp.

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<sup>1</sup> New York Agricultural Experiment Station Technical Bulletins, 1928.



# SOME CHEMICAL STUDIES OF COMMERCIAL BACTERIOLOGICAL PEPTONES

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## I. INTRODUCTION

In the course of a study of the abortus-melitensis group it was found that certain commercial peptones were better suited to initiate growth and multiplication than others. It was decided, therefore, to make as accurate chemical analyses as present methods will permit of certain well-known commercial brands of peptones in order to determine in what form nitrogen may be best utilized by the members of this bacterial group.

## II. METHODS

Four commercial brands of peptone were employed in this study: Fairchild's, Difco-Bacto, Difco-Proteose and Witte's.

One gram of the peptone, accurately weighed on a delicate balance, was dissolved in approximately 50 cc. of distilled water and sterilized in the autoclave for fifteen minutes at 15 pounds extra pressure. The peptone solution was carefully transferred to a 100 cc. volumetric flask and the whole made up to the mark. The following nitrogen determinations were made on aliquot portions of this solution. Five-cubic-centimeter amounts were used in all of the determinations except those for non-protein nitrogen and amino nitrogen by the Sørensen method where 20 cc. and 1 cc. quantities were used respectively.

*Total nitrogen.* By the Gunning modification of the Kjeldhal method.

*Non-protein nitrogen.* By the method of Folin and Wu.



*Free-ammonia.* By the Van Slyke and Cullen modification of the Folin air current process.

*Amino-nitrogen.* By the Van Slyke and Sørensen methods. The Sørensen results were corrected for free ammonia. The Van Slyke determinations were made on the ammonia-free material.

*Protein-nitrogen.* This was determined by subtracting the non-protein nitrogen from the total nitrogen figure.

*Polypeptid nitrogen.* This nitrogen fraction is represented as the difference between the non-protein nitrogen figure and the sum of the amino and free ammonia nitrogen. The Van Slyke figure was used here for the amino nitrogen.

Two different lots of each peptone were analyzed, and all determinations were made in duplicate. The figures obtained from the two different lots of the same brand of peptone were averaged. It was found that the different lots varied only slightly, the greatest variation being found in the two lots of Witte's peptone.

### III. EXPERIMENTAL

The results are given in the accompanying chart in which the different fractions are drawn to scale, with the exception of the free ammonia figures. Owing to the small amount of free ammonia, it was necessary to multiply the scale by ten in this case in order to make the results legible.

*Total nitrogen.* It will be seen that the amounts of total nitrogen do not vary very greatly in these four commercial peptones, Witte, with 152 mgm., is the highest, and Difco-Proteose, with 125 mgm., is the lowest.

*Non-protein nitrogen.* In the next figure on the chart are given the results of the non-protein nitrogen determinations. Witte's peptone which shows the largest amount of total nitrogen has the smallest amount of the non-protein fraction (56.17 mgm.). In the Difco-Bacto peptone the total nitrogen and non-protein nitrogen are not very far from equal. Difco-Proteose peptone has distinctly more non-protein nitrogen (69.3 mgm.) than Witte's, and in the Fairchild peptone, almost three-fourths of the total nitrogen is non-protein (102.5 mgm.).

*Protein-nitrogen.* These figures were not obtained by actual determination, but by the subtraction of the non-protein nitro-

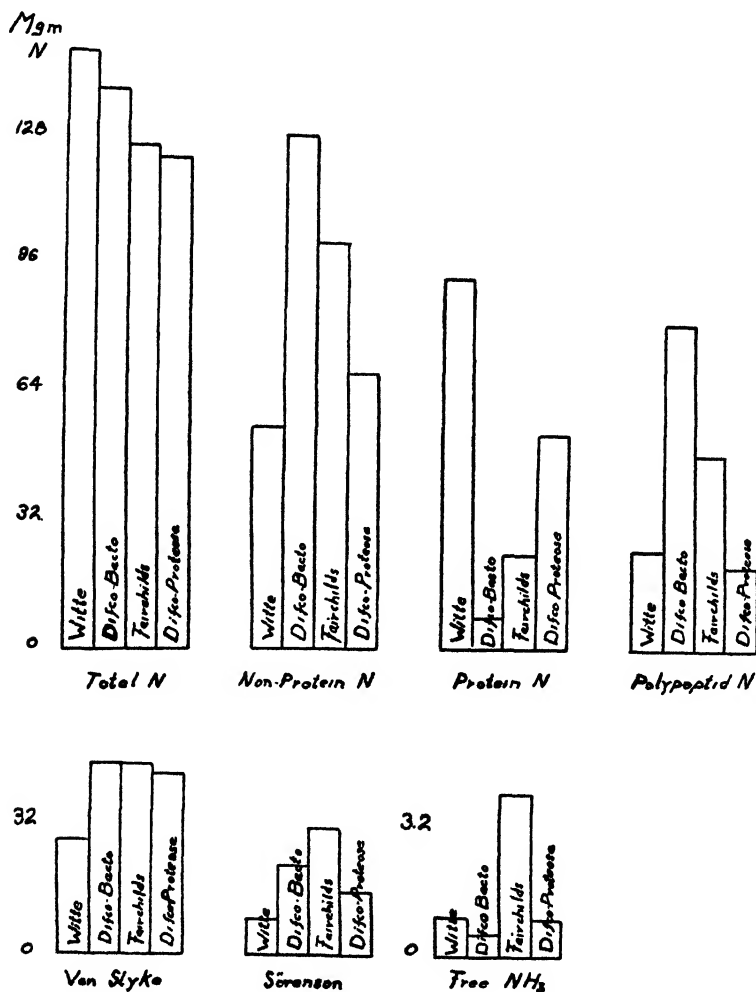


CHART 1

gen figures from the total nitrogen results. The chart shows that the Witte peptone contains the largest amount of protein nitrogen (93.42 mgm.), and that the Difco-Protease has next to the

largest (54.6 mgm.), though its protein nitrogen content is less than two-thirds that of the foreign brand. The protein nitrogen in Fairchild peptone is comparatively small (24.2 mgm.) and that in Difco-Bacto is almost negligible (8.4 mgm.).

*Polypeptid nitrogen.* This fraction also was not obtained by actual determination, but by subtracting the sum of the amino and free ammonia nitrogen figures from the amount of non-protein nitrogen. Difco-Bacto shows the largest amount of polypeptid nitrogen (82.17 mgm.) and Fairchild's next to the largest (79.57 mgm.). There was correspondingly much less polypeptid nitrogen in the Witte and Difco-Proteose peptones.

*Amino nitrogen.* By the Van Slyke determination the Difco-Bacto, the Difco-Proteose and the Fairchild peptone were found to have about equal amounts of amino nitrogen (approximately 48.0 mgm.), while the Witte peptone contained only about two-thirds as much (29.65 mgm.). The Sørensen results are more irregular, indicating perhaps that the di-amino acids were present in greater quantities in some of the brands than in others.

*Free ammonia.* With the exception of the Fairchild peptone, the amount of free ammonia in any of these peptones was almost negligible. It should be remembered that in the chart these amounts are drawn to a scale ten times greater than that used for the other determinations.

#### IV. DISCUSSION

The results given in this paper must be regarded merely as preliminary in the main. It is realized that if more lots of each of the peptones had been analyzed the relative amounts of the various fractions might have been expressed in somewhat different figures. However, as previously stated, the only large variations that were found were in the two lots of the Witte peptone.

Although the amounts of total nitrogen in all of these peptones appeared to be about equal, the different nitrogen fractions in the various brands differed greatly from each other. It is possible, in a measure at least, to estimate the length of the digestion period and the type of the enzyme employed in the production of peptone, from the above determinations. Relatively long diges-

tion with trypsin, particularly after continued preliminary treatment with pepsin, will of course yield relatively large amounts of non-protein, particularly amino, nitrogen. On the other hand, digestion with pepsin alone, or followed by a very short tryptic process, will furnish a product high in "protein" (proteose) nitrogen.

Practically all of the total nitrogen in the Difco-Bacto product was "non-protein" in character. There was a relatively large amount of polypeptids present, and according to the Van Slyke method the amino nitrogen fraction was high. There appeared to be very little free ammonia and "protein" nitrogen in this brand.

The non-protein nitrogen content of Witte's peptone was comparatively small, and was divided about equally between the polypeptid and amino nitrogen fractions. The amount of free ammonia was very small. A large portion of the total nitrogen was of the proteose type.

In the Difco-Proteose peptone the total nitrogen was about equally divided between the non-protein nitrogen and the protein nitrogen fractions. About one-third of the non-protein nitrogen was apparently polypeptid and two-thirds amino acid in character. The protein nitrogen was high and the free ammonia was practically negligible.

The Fairchild peptone contained a relatively large amount of non-protein nitrogen which was about equally divided between the polypeptid and amino nitrogen (Van Slyke) fractions. The amount of protein nitrogen was small. Evidently digestion is carried on for a longer time in the preparation of this peptone than with Witte and Difco-Proteose peptones. The free ammonia determinations were high.

If it may be assumed that the protein nitrogen is practically all proteose in character, the Witte product contains a very large quantity of this product. Difco-Proteose peptone has about half as much as Witte's, while the Fairchild and Difco-Bacto peptones contain relatively small amounts of this nitrogen partition. This is especially interesting in view of the more recent work showing that proteoses appear to be necessary for toxin production. Gibbs

and Rettger (1927) obtained potent toxins with both the Difco-Proteose and Witte peptone, and they showed that one fraction, evidently proteose in character, was necessary to obtain this result.

The results given above corroborate the statement made by Hucker and Carpenter (1927). "The original 'Witte peptone' contained a large amount of proteose and was only slightly digested in comparison with other brands. 'Fairchild's peptone,' on the other hand, is most completely digested. . . . 'Bacto peptone' appears to be intermediate between Witte and Fairchild's."

#### V. SUMMARY

1. Four different brands of commercial peptone were analyzed quantitatively for their various nitrogen fractions. Although the total nitrogen present in all of these different commercial brands was practically the same, there was a marked difference in the amounts of the various nitrogen fractions.

2. Witte and Difco-Proteose peptones contain the largest amount of so-called "protein" nitrogen. It is probable that most of this nitrogen is proteose in character.

#### REFERENCES

- GIBBS, C. S., AND RETTGER, L. F. 1927 Some factors governing the production of diphtheria toxin in artificial media. *Jour. Immunol.*, **13**, 323-344.
- HUCKER, G. J., AND CARPENTER, D. C. 1927 The relation of hydrolytic decomposition products of protein to bacterial growth. *Jour. Inf. Dis.*, **40**, 485-496.

# EXPERIMENTAL STUDIES OF BACTERIAL DEATH RATES IN POLLUTED WATERS

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It has been well known for many years that in sewage-polluted streams bacteria of the varieties included in the usual standard quantitative determinations tend to a quite consistent and rapid decrease in numbers. On the other hand, it is equally well known to bacteriologists that when waters from such streams are collected in artificial containers and observed in the laboratory, the usual tendency is to a very considerable increase in numbers of bacteria rather than to the decrease which is observed in the streams.

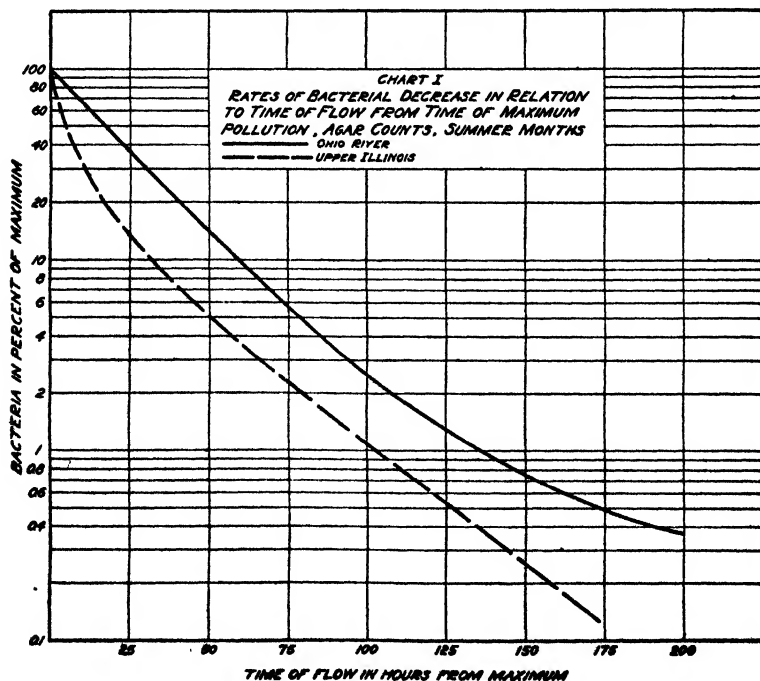
This wide divergence between the bacterial changes which take place in natural streams and those which take place in the same waters under usual laboratory conditions has to a large extent blocked progress in experimental studies which might serve to extend and clarify our knowledge of the factors concerned in bacterial purification in natural streams, for obviously no great progress is to be expected until we are able to reproduce under experimental conditions the changes which are known to take place in streams.

Recognizing the importance of experimental studies as an aid to the better understanding of the purification observed in streams, the Public Health Service laboratory for Stream Pollution Investigations, at Cincinnati, has been more or less constantly engaged, for the past ten years, in efforts to devise experimental methods which would serve this purpose. The laboratory is very fortunately situated for such studies, in that it has ready access to the Ohio River at, and immediately below, Cincinnati; and as a result of three years' intensive study of the river, making

TABLE 1

*Summary of bacteriological results, based on agar counts obtained during the summer season*

TIME OF FLOW  <i>hours</i>	OHIO RIVER		UPPER ILLINOIS RIVER	
	Bacteria per cubic centimeter	Bacteria in per cent of maximum	Bacteria per cubic centimeter	Bacteria in per cent of maximum
0	99,300	100.00	3,890,000	100.00
10	66,800	67.26	1,180,000	30.30
20	45,100	45.37	640,000	16.45
30	30,500	30.71	410,000	10.55
40	20,800	20.90	275,000	7.07
50	14,200	14.31	197,000	5.06
70	6,840	6.89	106,000	2.72
100	2,540	2.56	43,000	1.10
125	1,290	1.30	20,500	0.53
150	755	0.76	9,600	0.25
175	497	0.50	4,500	0.12
200	357	0.36	2,150	0.05



bacteriological examinations at successive cross sections, we already have fairly good information on the rate of bacterial purification which actually takes place in the river between Cincinnati and Louisville, that is, within a distance of about 130 miles, representing periods of flow from a few hours up to eight or ten days. Therefore, when samples of water are collected from the Ohio River in this vicinity and observed under experimental conditions, the changes noted can be compared quantitatively with those known to take place in the same body of water remaining in the stream.

The general course of bacterial decrease in the Ohio River below Cincinnati may be seen from table 1 and chart 1. These show, for successive periods of time, the percentage which the bacteria remaining in the river constitute of those originally present below the sewer outlets of Cincinnati. The curve shown is a somewhat smoothed average of observations made at various time intervals in spring, summer and autumn, at temperatures ranging from about 10 to 27°C. It refers only to agar plate counts, and in the discussion which follows only these need be considered, since the results in terms of gelatin plate counts or of *B. coli* are closely similar. The chart also shows, for purpose of comparison, the curve of bacterial decrease as observed in the Illinois River in the stretch from Lockport to Peoria, Illinois. The Illinois River in this stretch is much more grossly polluted than the Ohio, and it will be observed that the rate of bacterial decrease is much more rapid than in the Ohio but that the death rate curves are of the same general character in the two streams.

In the attempt to develop methods for studying natural purification on an experimental scale, four major set-ups have at various times been tried:

a. Samples have been stored in glass bottles in the incubator at constant temperatures.

b. Samples have been stored, with intermittent agitation, in wooden buckets at air temperatures.

c. Samples have been stored suspended in the river at the site of collection. And, within the past year,

d. The course of natural purification has been followed in water pumped from the river and detoured through artificial channels.



In all of these studies the water used has been collected from the Ohio River, at or below Cincinnati. Consequently the changes observed in this water under experimental conditions may properly be compared with those observed in the Ohio River at corresponding time intervals below Cincinnati.

#### A. SAMPLES STORED IN THE INCUBATOR

In this study, samples collected from the Ohio River at a fixed sampling station below the sewer outlets of the city were kept in

TABLE 2

*Summary of bacteriological results of 46 samples of Ohio river water (collected from section 475, just below Cincinnati metropolitan district), stored in bottles at 20°C.*

Results based on twenty-four-hour 37°C. agar counts

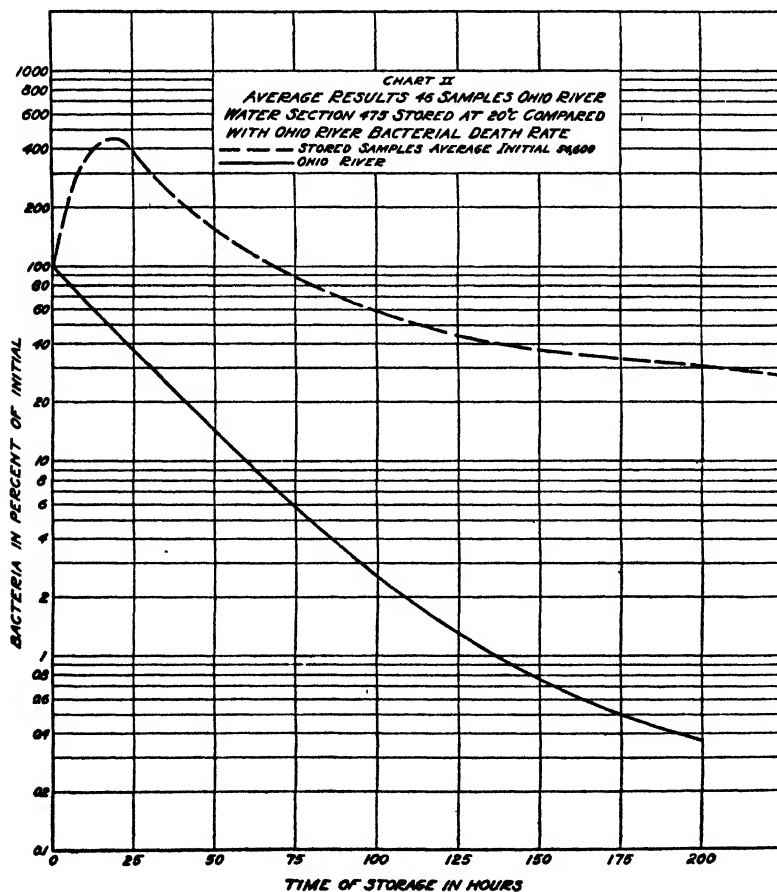
TIME OF STORAGE	BACTERIA PER CUBIC CENTIMETER	BACTERIA IN PER CENT OF INITIAL
<i>hours</i>		
0	54,600	100.0
4	99,600	182.0
8	164,000	300.0
20	244,000	447.0
24	216,000	396.0
48	90,000	166.0
72	50,500	92.5
144	15,700	38.8
312	8,620	15.8
960	2,580	4.72

the laboratory in ordinary sample bottles at various temperatures, namely at 10°, 20°, and 37°C., bacterial counts being made on each sample at intervals of four, eight, twenty, twenty-four, forty-eight hours, etc.

The observations thus made on 46 samples kept at a temperature of 20°C. are summarized in table 2 and chart 2. The chart also shows, for comparison, the rates of decrease actually observed in the same water in the Ohio River at ordinary summer and autumn temperatures.

In the data presented, two differences between river natural purification curves and the stored sample curves are observed:

1. There is a very marked increase in bacterial numbers in the stored samples during the first few hours of storage, and



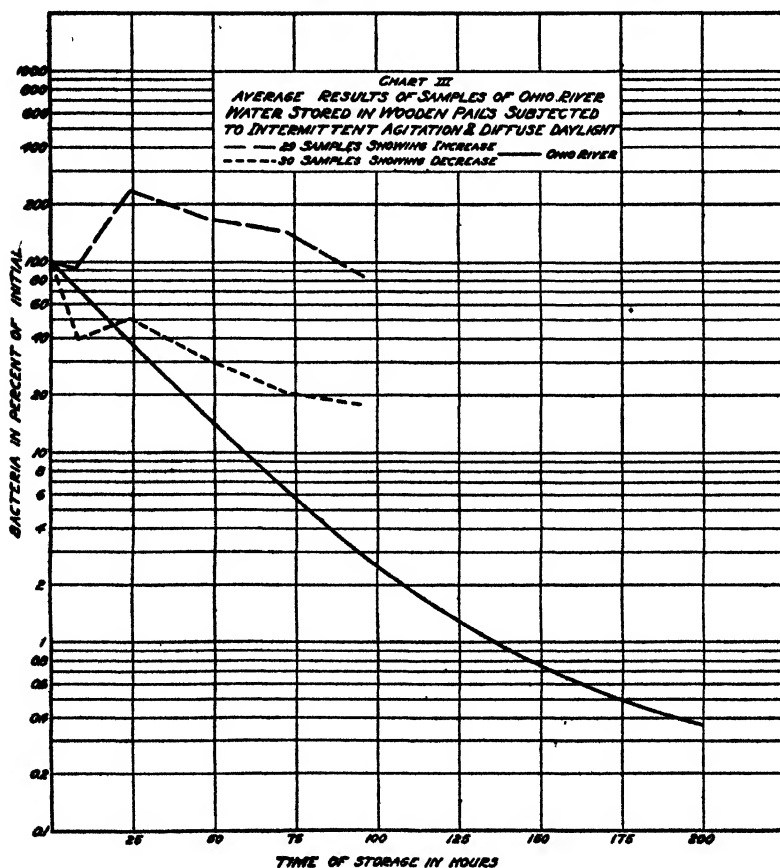
2. The subsequent rate of bacterial decrease is approximately the same for the forty-eight-hour period following the maximum, while thereafter the rate decreases rapidly.

TABLE 8

*Summary of bacteriological data from 69 samples of Ohio river water  
stored in wooden pails*

Results based on twenty-four-hour 37°C. agar counts

TIME OF STORAGE	RESULTS OF 29 SAMPLES SHOWING AN INITIAL INCREASE		RESULTS OF 30 SAMPLES IN WHICH NO INCREASE WAS OBSERVED	
	Bacteria per cubic centimeter	Bacteria in per cent of initial	Bacteria per cubic centimeter	Bacteria in per cent of initial
hours				
0	5,400	100.0	12,400	100.0
8	5,000	92.5	4,830	38.9
24	12,500	231.5	6,200	50.0
48	8,190	151.7	3,750	30.2
72	7,860	145.6	2,560	20.6
96	4,500	83.3	2,220	17.9



## B. SAMPLES STORED IN BUCKETS

To simulate more closely the conditions in a natural water-course, the next experimental set-up made use of thirteen wooden pails, each of about 3 gallons' capacity. Water from the Ohio River was passed through them in rotation from Pail 1 to 13. The transfer of water was made at eight-hour intervals. In sampling and transferring the water in the pails, the water in Pail 13 was mixed, sampled and wasted. The water in Pail 12 was then poured into Pail 13 and sampled. This process was continued

TABLE 4

*Summary of bacteriological results obtained from 73 samples of Ohio river water stored in glass bottles suspended in the river at the site of collection*

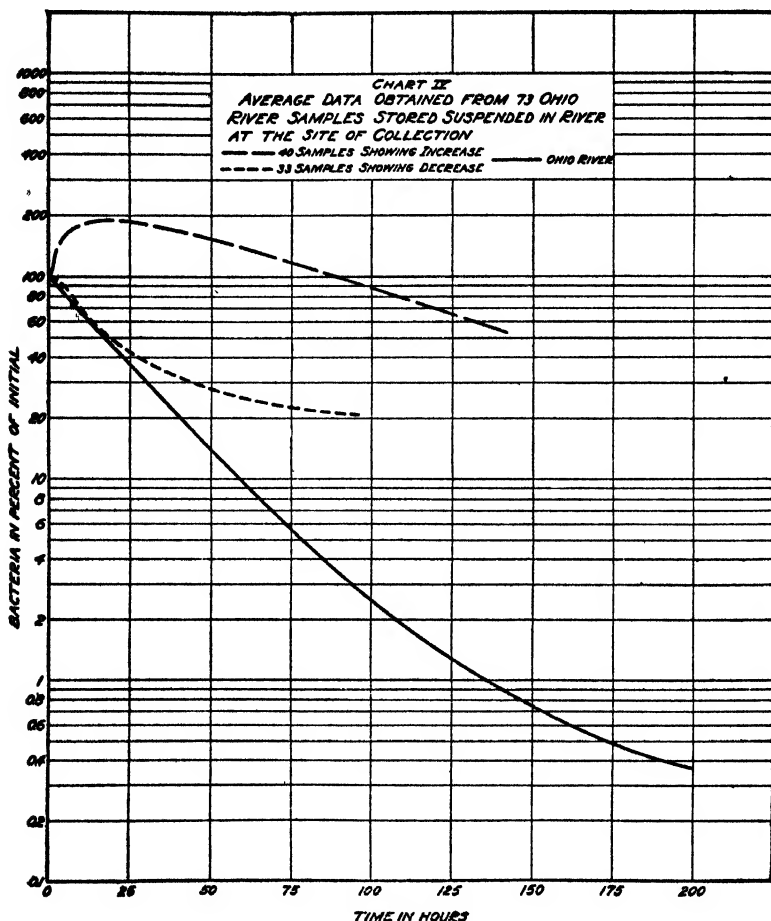
TIME OF STORAGE  <i>hours</i>	40 SAMPLES SHOWING INCREASE		33 SAMPLES SHOWING NO INCREASE	
	Average number of bacteria per cubic centimeter	Bacteria in per cent of initial	Average number of bacteria per cubic centimeter	Bacteria in per cent of initial
0	240,600	100.0	236,000	100.0
4	372,400	154.8		
8	415,300	172.6	181,000	76.7
12			131,900	55.9
24	447,700	186.1	106,900	45.3
48	337,600	140.3	66,300	28.1
72	327,700	136.2	53,800	22.8
96	197,800	82.2	49,800	21.1
120			14,200	6.0
144	129,000	53.6		

until Pail 1 was reached, which, each time it was emptied, was filled with fresh river water. The pails were stored in the open, exposed to diffuse daylight but not to sunlight.

Routine examinations were made and continued for thirty weeks, during which time the bacterial history of 59 samples was determined.

In 29 of the 59 samples stored thus in pails, no initial increase in bacterial numbers was found. In the remaining 30 samples, however, a very considerable increase was noted. The average results for the two groups are presented in table 3 and chart 3.

The results obtained indicate that we were able, at times, to reproduce the phenomena observed in the natural stream, but not consistently.



### C. SAMPLES SUSPENDED IN THE RIVER

In the third attempt to reproduce, under experimental conditions, natural purification as it occurs in streams, samples were collected and suspended in the stream at the site of collection, in order to avoid any change whatsoever in temperature and light

conditions. Various methods of storage were employed, using collodion sacs and glass bottles. The results obtained from samples stored in glass bottles are representative and the averages are presented in table 4 and chart 4 in two groups.

In this set-up the bacterial history of 73 samples was determined. There was a marked difference in the bacterial activity of the samples. In 40, a rather marked increase in bacterial numbers occurred. In 33, there was a similarly marked decrease. An intensive study of the conditions affecting each sample has failed to disclose any one factor or combination of factors which might be responsible for the difference.

#### D. SAMPLES FLOWING THROUGH ARTIFICIAL CHANNELS

In a further effort to reproduce experimentally the processes of natural purification occurring in streams, artificial channels have been constructed on the laboratory grounds. These channels are approximately eight-tenths of a mile in length and water has been passed through them with a time of flow of approximately two days. Bacteriological analyses have been made over a four months period of Ohio River water as it passed through these channels. In addition, bacteriological studies have been made of settled Ohio River water polluted with 5, 15, 30 and 45 per cent of fresh sewage as the mixtures passed through the channels.

A summary of the data obtained from these studies in the channels is given in table 5, and the results obtained from Ohio River water and from the 5 per cent concentration of sewage are graphically presented in chart 5.

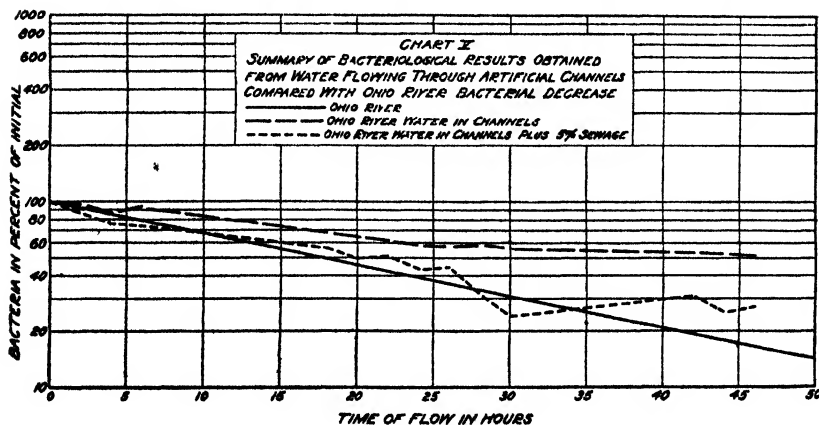
In the observations on death rates, in 15, 30 and 45 per cent concentrations of sewage in settled river water, two marked differences from natural conditions are noted:

- a. The initial rate of bacterial decrease is much more rapid in the channels, and
- b. It is always followed by a secondary rise.

It should be noted that with the 15, 30 and 45 per cent concentrations of sewage, the results available for averaging were less numerous than was the case with the 5 per cent sewage mixture

TABLE 5  
Summary of bacteriological results obtained from water flowing through  
artificial channels

TIME OF FLOW	OHIO RIVER WATER		ONE PART SEWAGE DILUTED WITH 19 PARTS SETTLED RIVER WATER		THREE PARTS SEWAGE DILUTED WITH 17 PARTS SETTLED RIVER WATER		SIX PARTS SEWAGE DILUTED WITH 14 PARTS SETTLED RIVER WATER		NINE PARTS SEWAGE DILUTED WITH 11 PARTS SETTLED RIVER WATER	
	Bacteria per cubic centi- meter	Per cent of initial	Bacteria per cubic centi- meter	Per cent of initial	Bacteria per cubic centi- meter	Per cent of initial	Bacteria per cubic centimeter	Per cent of initial	Bacteria per cubic centimeter	Per cent of initial
hours										
0	12,550	100.0	113,600	100.0	150,000	100.0	550,000	100.0	1,410,000	100.0
2	12,000	95.6	98,500	86.7	47,200	31.4	427,000	77.6	789,000	56.0
4	10,800	86.1	88,200	77.6	33,630	22.4	246,000	44.7	907,000	64.4
6	11,700	93.2	85,500	75.2	47,580	31.7	226,000	41.0	785,000	55.6
8					57,100	38.1	211,000	38.3	956,000	67.9
10					74,130	49.5	274,000	49.7	784,000	55.5
14					60,230	40.2	149,000	27.1	466,000	33.0
16					83,910	56.0	164,000	29.8	457,000	32.4
18	8,750	69.7	64,300	56.6	50,160	33.5	212,000	38.5	696,000	49.4
20	8,100	64.5	56,700	50.0	58,700	39.2	293,000	53.2	675,000	47.9
22	7,820	62.3	58,100	51.1	70,700	47.1	250,000	45.5	569,000	40.4
24	7,480	59.6	49,200	43.3	72,000	48.0	261,000	47.4	640,000	45.4
26	7,360	58.6	50,300	44.2	66,200	44.1	305,000	55.4	812,000	57.6
28	7,510	59.8	36,400	32.0						
30	7,120	56.7	27,700	24.4	54,100	36.1	235,000	42.7	694,000	49.2
34					51,500	34.4	451,000	82.0	1,120,000	79.5
38					41,000	27.4	462,000	84.0	756,000	53.7
42	6,780	54.0	35,500	31.2	40,900	27.3	515,000	93.5	673,000	47.8
44	6,750	53.8	29,160	25.6						
46	6,480	51.6	30,960	27.2	50,700	33.8	391,000	71.0	860,000	61.0



and particularly with Ohio River water. A longer series of results would probably make the curves more regular but would not be expected to affect the general trend.

#### SUMMARY

Data now available define fairly well the course of natural purification in certain stretches of the Ohio and Illinois Rivers. Samples stored in bottles at definite temperatures yield a bacterial history which varies from that in the stream in two ways:

- a. A marked initial increase in bacterial numbers occurs, and
- b. The final decline in bacterial numbers is not nearly as rapid in the bottles as in the river.

However, for the forty-eight-hour period, following the time of the maximum count in the bottles, the bacterial death rate very closely resembles that observed in the stream during the same period.

The bacteriological results obtained from samples stored in wooden pails and in glass bottles suspended in the river are variable and do not at all agree with those obtained in the stream.

In the study of bacterial death rates in water flowing through the artificial channels, the rates obtained for a forty-six-hour period, from Ohio River water and from settled river water polluted with 5 per cent of fresh sewage, simulate those observed under natural conditions.

In conclusion, it is not desired to create the impression that an experimental set-up has been devised which will reproduce consistently the processes of natural purification as observed in streams. It is believed that the experimental set-up described produces results which simulate those observed under natural conditions. It is possible that with further study and with some variations in the channels, results may be obtained which will agree uniformly with the data obtained from natural streams.





# SOME OBSERVATIONS ON THE PLATE-COUNT METHOD OF ENUMERATING BACTERIA IN MILK

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A large number of both scientific and semi-popular papers have been written dealing with the plate-count method of enumerating the bacteria in milk. The greater number of these papers are derogatory to this method and consequently are tending to destroy confidence in it. Extensive personal experience with the plate-count method of enumeration has led the writer to believe that the severity of the criticisms offered is unwarranted.

It is desired in this paper to discuss certain possible sources of the "gross discrepancies" that have been reported in plate counts of milk and to demonstrate that careful technique is important and that a consideration of the principles involved in bacterial growth is essential where agreement in plate counts is desired.

Two types of experiments have commonly been used to demonstrate variations in plate counts on the same sample of milk. In one type the practice has been to pour a large number of plates, usually 100, from the same sample of milk; in the other, portions of the same sample have been plated by workers in separate laboratories. In both types of experiment the counts obtained were used as a basis for judging the accuracy and reliability of the plate-count method. Extremely wide variations have been reported. Wright and Thornton (1927), for example, in reporting upon data obtained on counts of 2,330 plates, found discrepancies as great as 1,500 per cent under conditions of "extreme care." They state further that "a variation within a series, of less than 100 per cent, can not be depended upon." Similar discrepancies

have been reported by workers employing the two methods above mentioned. It has been stated that these differences have been due to the limitations of the plate-count method.

The efficiency of any determinative method depends upon the skill and care with which it is carried out. It is difficult to understand how such variations in plate counts on the same sample of milk as have been repeatedly reported could occur if the technique employed were consistent with the accuracy required of the method. It has been found, however, that the technique used in many experiments was governed by such factors as the conditions of the experiments, laboratory conditions, laboratory methods, and practicability. The effect upon the plate counts of such factors as accuracy and cleanliness of glassware, care in manipulation, contamination, methods of incubation, and counting, has frequently been discussed. It seems well, however, to point out that when the reliability of a method is to be judged, every factor contributing to its accuracy should be given full consideration.

Where several workers are employed on one experiment, a situation common to both of the test methods under discussion, potential sources of error enter. Personal errors are incalculable. Personal idiosyncrasies, differences in technique, and individual interpretation of values, all contribute to irregular results. In the experiments where a number of workers are engaged independently and in different laboratories in making plate counts on the same sample of milk, there is in addition no coördination of time or exposure or even in the manner of handling the samples. Too little consideration has been given, in both experimental methods, to these sources of error.

Comparisons of plate counts with direct microscopic counts obtained by the Breed method have shown extremely wide variations. This is to be expected because counts obtained by the Breed method include both the living and the dead cells. While this is an elementary criticism, it is nevertheless sound and receives support from the work of Wilson (1922), who demonstrated that even in the logarithmic phase of growth, and under optimum conditions, the number of living cells rarely exceeds 90 per cent

of the total cell population. Other sources of error exist in the Breed method and are generally apparent, yet the Breed counts have frequently been used as a standard upon which to gauge the reliability of the plate-count method.

In making plate counts of milk living organisms are being dealt with. Sherman and Albus (1923) demonstrated that young bacterial cells were more susceptible to certain environmental hazards than were old and mature cells and suggested the possible effect of this susceptibility upon bacterial enumeration. For example, it was shown that exposure to heat or cold caused a marked mortality among growing cells, whereas similar exposures had little or no effect upon old or mature cells. The application of these findings to bacterial enumeration is readily apparent. A sample of milk in which bacteria are actively growing, if packed in ice for a period of time, shows a wide discrepancy in count when both the plate and the Breed methods are used. This discrepancy is due to the fact that many of the more sensitive or young cells are killed by the chilling. The temperature of the water blanks, the time of exposure therein, and the temperature of the agar, likewise have their effect upon young growing cells. A disregard for environmental hazards when making comparative counts is unfair to the plate method, and variable results must be expected.

Bacterial growth may cause wide variations in plate counts. The rapidity of growth of bacteria under favorable conditions has long been known, yet the possible effect of this factor upon plate counts appears generally to be ignored. The importance of a possible increase in the number of bacteria, during the plating process, is given recognition in "Standard Methods of Milk Analysis" (1927) which directs that the plating process is to be completed within fifteen minutes. The time consumed in the plating process is especially important when consistent plate counts are demanded. Considerable time must be consumed in making a large number of plates from the same sample, and a disagreement in count may be expected. The general disregard for such factors as time and growth can readily be appreciated when consideration is given to the manner of making plate counts

which prevails in public-health and other laboratories where daily counts are made on a number of samples of milk.

#### EXPERIMENTAL

The following experiments are reported to demonstrate the possible effect on plate counts of the factors of time, temperature, and growth. Data representative of the results obtained in many experiments are presented in tables 1 and 2.

The procedure employed in the experiments reported was as follows:

TABLE 1

*The effect of time, temperature, and growth on the results obtained by the plate method on milk of low bacterial content*

	PLATED IM- MEDIATELY	AGAR POURED AFTER 15 MINUTES	HELD IN DILUTION BOTTLE 15 MINUTES
Original count .....	{ 5,300 5,900		
Held at room temperature for 2 hours .....	{ 13,200 13,500	16,600 16,000	18,000 19,200
Held in ice for 2 hours .....	{ 10,000 9,000	10,000 9,300	15,400 16,500
Held at room temperature for 30 minutes .....	{ 17,700 17,800	23,800 24,500	29,000 28,300

Platings were made of each sample of milk immediately after it was received. The sample was warmed to room temperature and held for two hours. At the end of this time the bacteria present in the milk had begun active multiplication, and a second plating was made. The sample was then packed in ice for two hours and at the end of this period a third plating was made. Following this the sample was again warmed to room temperature and held for thirty minutes after which a fourth plating was made.

Six plates were poured from the same dilution at each plating with the exception of the first plating shown in table 1. A measured amount was poured, by means of a pipette, into each of

four plates at the same time. The agar was poured into two of these plates immediately and into the remaining two plates fifteen minutes later. Two plates were poured from the same dilution bottle after it had stood at room temperature for fifteen minutes. Thus there were three duplicate sets of plates made of each milk sample. An inspection of the tables will indicate more clearly the procedure outlined.

The following precautions were taken to obtain uniform results: Previous to sterilizing, all pipettes were washed in a cleansing

TABLE 2

*The effect of time, temperature, and growth on the results obtained by the plate method on milk of high bacterial content*

	PLATED IMMEDIATELY	AGAR POURED AFTER 15 MINUTES	HELD IN DILUTION BOTTLE 15 MINUTES
Original count .....	6,000,000 5,400,000	6,200,000 7,500,000	7,700,000 6,000,000
Held at room temperature for 2 hours .....	28,000,000 28,100,000	35,700,000 34,000,000	35,800,000 35,600,000
Held in ice for 2 hours .....	31,800,000 30,000,000	30,800,000 29,200,000	33,400,000 33,900,000
Held at room temperature for 30 minutes ...	45,700,000 45,800,000	46,800,000 45,000,000	48,000,000 50,800,000

solution and thoroughly rinsed in water. Only one delivery was made from a single pipette, because it has been observed many times when plating whole milk, especially from the first dilution bottle, that a quantity of material clings to the inside of the pipette when a second delivery is attempted. This is a source of error which has probably been frequently overlooked when checking plate counts. For example, Prescott and Parker (1927), to preserve uniformity and accuracy, employed the same pipette in pouring 10 plates from an identical 1:100 dilution of milk, and in their summary call attention to "the inherent inaccuracy of any plate method of bacterial enumeration. . . ."

Dilutions were made in 99 cc. blanks of distilled water which were at room temperature. Standard beef extract agar was used and poured at a temperature of 42°C. After the plates were poured they were placed immediately on a cold plate until the agar had hardened and were subsequently removed before they could become chilled. All manipulations were carried out as quickly as possible and with the greatest care. The plates were incubated for two days at 37°C.

It will be observed from the tables that an appreciable increase in count resulted when the plates were held at room temperature for fifteen minutes before pouring the agar and that a still greater increase resulted from holding the dilution blanks at room temperature for fifteen minutes before completing the plating process. It is recognized that the increases in count may be due to a breaking up of groups or clumps of bacteria because of some unknown action resulting from their being suspended in distilled water—at the same time these increases are observed only when the bacteria are multiplying. In table 2 the first series of counts, which were made before active bacterial growth had begun in the sample, shows practically no increase in number when plates or the dilution blanks were held at room temperature for fifteen minutes. It is believed that lack of appreciation of such changes may be responsible, in a large measure, for the irregular counts often obtained by the plate method.

Table 1 shows clearly what takes place when a sample of milk in which the bacteria are growing is packed in ice. The number of living bacteria in the sample may be reduced materially by this procedure. While a temporary dormancy of the cells may be brought about by the chilling, it is also shown in the tables that when the sample has been held at laboratory temperature for thirty minutes, sufficient growth has taken place to affect the count materially and that the bacteria are again sensitive to the time and temperature hazards of the plating process.

It is to be noted from the data in both tables that the differences in counts due to the various factors employed are not of the same magnitude. This is to be expected when the difference in the bacterial content of the two samples is recognized. When the

bacterial content is low, growth proceeds rapidly and unimpeded, but when it is high, growth is hindered by the accumulated retarding factors that limit cell population. It must be apparent from the data presented in the tables that duplicate plate counts of the same sample of milk can be made that will agree so closely as to satisfy the most exacting bacteriologist, provided all possi-

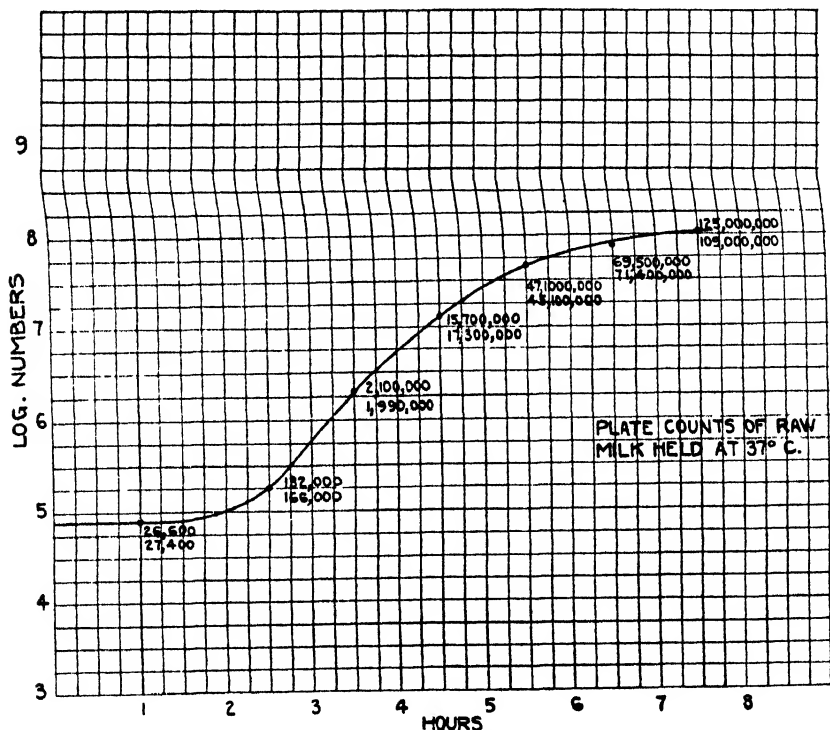


FIG. 1. BACTERIAL GROWTH IN RAW MILK. PLATE COUNT METHOD

ble sources of error are controlled to the limit permitted by the method.

There is no known method of accurately enumerating bacteria. It appears, however, that the comparability of plate counts and the reliability of the plate-count method of enumeration can be demonstrated, if the counts obtained by successive platings of a sample of milk in which the bacterial population is progressively



changing give a smooth and characteristic curve when they are plotted.

Figure 1 represents a growth curve obtained by plotting, against time, the logarithms of the number of living bacteria as determined by the counts obtained on a quantity of raw milk held at 37°C. until curdled. The uniform character of the curve is submitted as evidence of the reliability of the plate-count method when it is carefully employed. The severity of the technique used in this experiment to determine the efficiency of the plate-count method can better be appreciated when consideration is given to the fact that the curve was obtained on the mixed bacterial flora of raw milk. A similar curve is easily obtainable when a pure culture is being plated.

As an aid to those who may be unfamiliar with a logarithmic curve, the actual plate counts are shown in the figure.

#### DISCUSSION

No method of accurately enumerating bacteria has yet been found. Accuracy in bacterial enumeration is highly improbable if not impossible. As far as the writer has been able to find, the claim that bacteriology is an exact science has been set up by those desiring to demolish it. Methods of bacterial enumeration are methods of approximation. The plate-count method when conscientiously employed by trained personnel can, and does, give consistent counts when the sources of all possible error are fully controlled. The looseness of the methods generally practiced in making plate counts would be unthinkable in any chemical analysis, yet analytical results are expected. The assertion that widely discrepant plate counts are due to errors inherent in the plate method is an admission rather than an argument. The methods used in carrying out the experiments on which this assertion is based have been unnecessarily involved. Needful precautions when preparing plates have not been observed, and additional sources of error have been introduced.

The data presented in this paper bear out the contention of the writer, based on extensive experience with the plate-count method,

that when proper care is exercised comparable plate counts can be obtained.

#### SUMMARY

1. It is pointed out that the experimental methods employed to obtain the data upon which plate counts of milk have been so severely criticized, permit errors that may be responsible for the resulting discrepant counts.

2. It is demonstrated that when a sample of milk in which bacteria are actively growing is packed in ice for a period of time, a number of the organisms are killed.

3. The time consumed in the plating process will markedly affect the accuracy of the plate counts if the bacteria are actively multiplying, especially in milks of relatively low bacterial content.

4. It is demonstrated that with the proper care and a consideration of all the factors involved consistent plate counts of milk can be made.

#### REFERENCES

- PRESCOTT, S. C., AND PARKER, M. E. 1927 Use of blood agar in control of milk supplies. 16th Ann. Rept. Int. Assoc. Dairy and Milk Inspectors.
- SHERMAN, J. M., AND ALBUS, W. R. 1923 Physiological youth in bacteria. *Jour. Bacteriol.*, 8, 127.
- Standard Methods of Milk Analysis. 1927. 5th ed. American Public Health Association.
- WILSON, G. S. 1922 The proportion of viable bacteria in young cultures, with special reference to the technique employed in counting. *Jour. Bacteriol.*, 7, 405.
- WRIGHT, W. H., AND THORNTON, H. R. 1927 How accurate is the quantitative plate count? (Abstract.) *Jour. Bacteriol.*, 13, 63.



# CHEMICAL CONSTITUTION AND GERMICIDAL ACTIVITY OF AMINES, KETONES, AND ALDEHYDES

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In two preceding papers the writers (Tilley and Schaffer, 1926; Schaffer and Tilley, 1927) have discussed the relation between the chemical constitution and the germicidal activity of various alcohols and phenols. The present paper reports the results of similar investigations concerning some of the alkyl and aryl amines and some of the alkyl ketones and aldehydes.

## MATERIALS

Most of the compounds studied were obtained from commercial sources. Those which could not be thus procured were made in these laboratories. Whatever the source, however, all of the substances, with the exception of formaldehyde, were carefully purified. The formaldehyde was a standardized solution of a commercial sample of formalin. Because of the small quantities available, the n-hexylamine and n-heptylamine made by us were collected over a wider boiling point range than was permitted with the other compounds. The compounds used and the boiling points or solidification point are recorded in table 1. The boiling points were determined by means of standardized Anschütz thermometers, the stems of which were immersed in the vapors of the liquid during distillation. The table of Young (1902) was used in correcting boiling points for changes in barometric pressure.

## BACTERIOLOGICAL WORK

For the purposes of the present investigation, just as in our previous work, the germicidal efficiency of the compounds studied

has been measured by the phenol coefficients, determined by a modified Rideal-Walker method. The exact technique has already been described, (Tilley and Schaffer, 1926) but we wish again to emphasize the fact that the coefficients determined by this method are based on the relative amounts by weight of the

TABLE 1  
*Physical constants of substances tested*

SUBSTANCE	FORMULA	BOILING POINT °C.
Methyl methyl ketone.....	$\text{CH}_3\text{COCH}_3$	56.2-56.5
Methyl ethyl ketone.....	$\text{CH}_3\text{COC}_2\text{H}_5$	79.5-79.8
Methyl n-propyl ketone.....	$\text{CH}_3\text{COC}_3\text{H}_7$	102-103
Methyl n-butyl ketone.....	$\text{CH}_3\text{COC}_4\text{H}_9$	127-128
Methyl n-amyl ketone.....	$\text{CH}_3\text{COC}_5\text{H}_{11}$	150.5-151.1
n-Propylamine.....	$\text{C}_3\text{H}_7\text{NH}_2$	48-50
n-Butylamine.....	$\text{C}_4\text{H}_9\text{NH}_2$	77-78
n-Amylamine.....	$\text{C}_5\text{H}_{11}\text{NH}_2$	104-106
n-Hexylamine.....	$\text{C}_6\text{H}_{13}\text{NH}_2$	125-135
n-Heptylamine.....	$\text{C}_7\text{H}_{15}\text{NH}_2$	155-165
Diethylamine.....	$(\text{C}_2\text{H}_5)_2\text{NH}$	55-56
Di-n-propylamine.....	$(\text{C}_3\text{H}_7)_2\text{NH}$	109-110
Di-n-butylamine.....	$(\text{C}_4\text{H}_9)_2\text{NH}$	159-161
Triethylamine.....	$(\text{C}_2\text{H}_5)_3\text{N}$	89.5-90.2
Benzylamine.....	$\text{C}_6\text{H}_5\text{CH}_2\text{NH}_2$	185-186
Aniline.....	$\text{C}_6\text{H}_5\text{NH}_2$	184.5-185.0
Paratoluidine.....	$\text{CH}_3\text{C}_6\text{H}_4\text{NH}_2$	*
Methyl aniline.....	$\text{C}_6\text{H}_5\text{NHCH}_3$	195.5-196.2
Ethyl aniline.....	$\text{C}_6\text{H}_5\text{NHC}_2\text{H}_5$	204.7-205.0
Formaldehyde.....	$\text{HCHO}$	
Acetic aldehyde.....	$\text{CH}_3\text{CHO}$	22.5-23.0
Propionic aldehyde.....	$\text{C}_2\text{H}_5\text{CHO}$	48.8-49.5
Butyric aldehyde.....	$\text{C}_3\text{H}_7\text{CHO}$	75-76

\* Solidification point 43.4°.

disinfectant and of the phenol standard required to kill the test organism in the same length of time.

In addition to the phenol coefficients there are also shown what are designated as "molecular" coefficients. As explained in our first paper these are the phenol coefficients converted from the original gram weight basis to a gram molecular basis.

Results obtained with the amines are shown in table 2. In the

columns headed "Ratios" there are shown the ratios between the molecular coefficients of successive members of each series of compounds. It will be seen that with *B. typhosus* as the test

TABLE 2  
*Coefficients of amines*

NAME	PHENOL COEFFICIENT	MOLECULAR COEFFICIENT	RATIO
Test organism, <i>B. typhosus</i>			
n-Propylamine.....	1.6	1.0	1.6
n-Butylamine.....	2.1	1.6	1.7
n-Amylamine.....	3.0	2.8	1.7
n-Hexylamine.....	4.5	4.8	1.7
n-Heptylamine.....	6.5	8.0	
Diethylamine.....	2.2	1.7	
Di-n-propylamine.....	2.0	2.2	
Di-n-butylamine.....	4.7	6.4	
Triethylamine.....	1.5	1.6	
Benzylamine.....	1.0	1.1	
Aniline.....	0.57	0.56	2.5
p-Toluidine.....	1.25	1.42	
Methylaniline.....	1.8	2.0	2.0
Ethylaniline.....	3.0	3.9	
Test organism <i>Staph. aureus</i>			
n-Propylamine.....	0.23	0.14	2.9
n-Butylamine.....	0.52	0.40	3.0
n-Amylamine.....	1.3	1.2	2.8
n-Hexylamine.....	3.1	3.3	
Diethylamine.....	0.21	0.16	
Triethylamine.....	0.25	0.27	
Aniline.....	0.50	0.49	

organism the average ratio between coefficients of the primary alkylamines is approximately 1.7, while with *Staph. aureus* as the test organism the average ratio is approximately 2.9. With

respect to the amines of the other series studied it was found that the relative insolubility of the members of these series rendered it impracticable to obtain coefficients except for the lower members. Such ratios as are shown can be regarded as only very roughly indicative of the average ratios for each different series, since our previous work indicates that ratios for the lowest members of a series are apt to be lower than the average for the entire series.

Previous experience having shown that great variations in results are sometimes obtained with different strains of the same test organism, tests were made upon primary n-butylamine and primary n-amylamine with strains of *B. typhosus* and *Staph.*

TABLE 3  
*Comparative results with different strains of B. typhosus and of Staph. aureus*

TEST ORGANISM AND STRAIN	MOLECULAR COEFFICIENT		RATIO
	n-Butylamine	n-Amylamine	
<i>B. typhosus</i> No. 2.....	1.0	2.3	2.3
<i>B. typhosus</i> No. 3.....	1.55	3.22	2.1
<i>B. typhosus</i> No. 4.....	1.55	3.22	2.1
<i>B. typhosus</i> No. 5.....	1.4	2.85	2.0
<i>Staph. aureus</i> No. 2.....	0.32	1.0	3.1
<i>Staph. aureus</i> No. 3.....	0.39	1.4	3.6
<i>Staph. aureus</i> No. 4.....	0.47	1.66	3.5

*aureus* other than those previously employed. The results of these comparative tests are shown in table 3, and, although there are differences in the results with different strains, the average ratio with all strains of *B. typhosus* is not far from 2.0 while with all strains of *Staph. aureus* the average ratio is approximately 3.3.

Results obtained with a number of alkyl ketones are shown in table 4. Results with *Staph. aureus* were limited to those for the lower members of the series because the relative insolubility of the higher members rendered it impossible to get enough of the disinfectant in solution to kill the test organism within the time limits of the method of testing employed. It is very interesting to note that the phenol coefficient of each member of the

ketone series is practically identical with that of the member of the primary alcohol series which contains one less carbon atom. The coefficients of the primary normal alcohols have already been

TABLE 4  
*Coefficients of alkyl ketones compared with those of primary alcohols*

ALCOHOLS	PHENOL COEFFICIENT	MOLECULAR COEFFICIENT	KETONES	PHENOL COEFFICIENT	MOLECULAR COEFFICIENT	RATIO	CALCULATED MOLECULAR COEFFICIENTS*
Test organism <i>B. typhosus</i>			Test organism <i>B. typhosus</i>				
Ethyl.....	0.04	0.020	Methyl methyl .....	0.04	0.025	3.1	0.025
Propyl.....	0.102	0.065	Methyl ethyl .....	0.102	0.078	3.2	0.078
Butyl.....	0.273	0.215	Methyl n-propyl.....	0.275	0.25	3.3	0.25
Amyl.....	0.78	0.73	Methyl n-butyl.....	0.78	0.83	3.4	0.83
Hexyl.....	2.3	2.5	Methyl n-amyl.....	2.3	2.8		2.8
Test organism <i>Staph. aureus</i>			Test organism <i>Staph. aureus</i>				
Ethyl.....	0.039	0.019	Methyl methyl.....	0.036	0.022	2.8	0.024
Propyl.....	0.092	0.053	Methyl ethyl .....	0.080	0.061		0.063

\* Calculated from molecular coefficients of alcohols by use of the formula:

$$\text{Mol. coeff. of ketone} = \frac{(\text{mol. wt. of ketone}) (\text{mol. coeff. of alcohol})}{\text{mol. wt. of alcohol}}$$

TABLE 5  
*Coefficients of aldehydes*  
Test organism *B. typhosus*

NAME	PHENOL COEFFICIENT	MOLECULAR COEFFICIENT
Formaldehyde.....	1.05	0.34
Acetaldehyde.....	0.09	0.04
Propionic aldehyde.....	0.15	0.09
Butyric aldehyde.....	0.32	0.25

reported in our first paper and are included in table 4 for convenient reference. The slight differences between the molecular coefficients of the alcohols and those of the ketones are due to



differences in the respective molecular weights. It is possible to calculate the molecular coefficients of the ketones from those of the alcohols by correcting for these differences according to the following formula:

$$\text{Mol. coeff. of ketone} = \frac{(\text{mol. wt. of ketone}) (\text{mol. coeff. of alcohol})}{\text{mol. wt. of alcohol}}$$

Coefficients calculated by this formula are shown in table 4 in the column headed "Calculated."

Results obtained with a few of the aldehydes are shown in table 5. It was difficult to obtain consistent results, but those shown will indicate with a fair degree of accuracy the relative germicidal values against *B. typhosus*.

#### DISCUSSION

The relation between chemical constitution and germicidal activity expressed by the ratios between molecular coefficients reported in this paper is closely parallel to the relation between chemical constitution and *antiseptic* activity deducible from the results reported by Kligler (1918), to which Bachman (1928) has recently called attention. If the minimum inhibitory concentrations given by Kligler (1918), for *B. typhosus* are reduced to a gram-molecular basis the ratio between concentrations for successive members of each series is observed to be not far from 2.0. For example, with aniline and para toluidine the actual amounts by weight required to produce inhibition in 5 cc. of the culture medium, expressed on a molar basis, are 0.0001537 and 0.000084, respectively, and the ratio between them is  $\frac{0.0001537}{0.000084} = 1.8$ .

In like manner with methyl aniline and ethyl aniline the respective weights are 0.000072, and 0.0000375 yielding a ratio of 1.9. In the case of dimethyl aniline and dimethyl p-toluidine the weights in 5 cc. are 0.000027 and 0.0000126, yielding a ratio of 2.1.

In view of the discrepancy between the above-mentioned ratio of 1.8 for aniline and para toluidine and our own ratio of 2.5 for these compounds, we have carefully repeated the work, but have obtained the same results. In this connection it may be men-

tioned that the coefficients reported by us in this paper for aniline and para toluidine agree completely with those reported by Morgan and Cooper (1912).

#### SUMMARY

In continuation of their studies of the relation between the chemical constitution and the germicidal activity of various classes of organic compounds the authors have determined the phenol coefficients of a number of alkyl and aryl amines and alkyl ketones and aldehydes.

Results obtained with primary alkylamines indicate that with *B. typhosus* as the test organism the average ratios between successive molecular coefficients vary somewhat with different strains, the general average ratio with all strains being approximately 2.0. With *Staph. aureus* as the test organism the ratios also vary somewhat with different strains but the general average ratio with all strains is approximately 3.3.

Results obtained with alkyl ketones indicate that with *B. typhosus* as the test organism the average ratio between successive molecular coefficients is 3.25.

Results with the aldehydes were not such as to yield a satisfactory ratio for the series, although they suggest that this ratio may be 2.5 or more.

#### REFERENCES

- BACHMAN, G. W. 1928 Chemical constitution and germicidal activity. *Proc. Soc. Exp. Biol. and Med.*, **25**, 249-250.
- KLIGLER, I. J. 1918 A study of the antiseptic properties of certain organic compounds. *Jour. Exp. Med.*, **27**, 463-478.
- MORGAN, G. T., AND COOPER, E. A. 1912 The influence of the chemical constitution of certain organic hydroxyl and aminic derivatives on their germicidal value. 8th Int. Cong. Applied Chem., **19**, 243-257.
- SCHAFER, J. M., AND TILLEY, F. W. 1927 Further investigations of the relation between the chemical constitution and the germicidal activity of alcohols and phenols. *Jour. Bacteriol.*, **14**, 259-273.
- TILLEY, F. W., AND SCHAFER, J. M. 1926 Relation between the chemical constitution and germicidal activity of the monohydric alcohols and phenols. *Jour. Bacteriol.*, **12**, 303-309.
- YOUNG, SYDNEY 1902 Correction of boiling points of liquids from observed to normal pressure. *Jour. Chem. Soc.*, **81**, 777-783.



# THE EFFECT OF PURE SOAPS ON THE BACTERICIDAL PROPERTIES OF PHENOLIC GERMICIDES

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## INTRODUCTION

It has been abundantly shown that soaps in general have a limited germicidal value, (for complete bibliography see Walker, 1924, 1925, 1926, and Eggerth, 1927), and various workers have expressed the idea that this is due for the most part to their detergent properties. The literature indicates that a large variety of organisms have been tested, at time intervals of exposure from two and one-half to sixty minutes. Soaps prepared from various types of oils have shown more or less selective action. The typhoid, diphtheria and colon bacilli, the spirochetes, the gonococcus, the meningococcus and different types of the streptococci are easily destroyed. On the other hand, it is significant that *Staphylococcus aureus* is particularly resistant. It is no doubt because of this selective action that efforts have been made from time to time to incorporate certain germicidal substances into soaps, and thereby manufacture a product which will not only cleanse but at the same time destroy organisms found on the skin, regardless of the type present. There are numerous commercial soaps on the market at the present time, to many of which have been added phenolic derivatives. The soaps are labelled as antiseptic, and a phenol coefficient is often determined. As yet there are no scientific data to show that the incorporation of the phenolic substances has added to the intrinsic germicidal activity which the soap itself may possess. The determination of the

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phenol coefficient for such a so-called "antiseptic soap" is in practice the measure of its activity against *Bacillus typhosus*, which organism has been shown by several workers to be very susceptible to the disinfectant action of soaps.

Hamilton (1917) mixed cresylic acid and soap in varying proportions, and reported that when the ratio between the two substances was varied to obtain in the one case a complete solution and in the other a hazy emulsion, the two preparations failed to show any material difference in germicidal effectiveness. When one examines the table given, however, it is seen that the efficiency was actually diminished by one-half when the amount of disinfectant was reduced from 80 to 50 per cent and the soap increased from 20 to 50 per cent. Tilley and Schaeffer (1925) found that the bactericidal efficiency of cresol was diminished in linseed oil soap-cresol solutions if more than half the mixture was soap. The present author, at the suggestion of Frobisher, has shown the futility of mixing large quantities of soap with so powerful a germicide as hexyl resorcinol (see Frobisher, 1927). Rettger, Valley and Platridge (1928) found that butyl resorcinol loses a part of its germicidal properties in the presence of pure soaps.

This investigation was undertaken to determine the effect of pure individual soaps on the germicidal activity of certain representative phenolic substances. Since the exact germicidal titre of pure soaps is easily obtained, it was possible to use the soaps in such low concentrations that the bactericidal effectiveness of the germicides was not masked by the disinfectant action of the soaps themselves.

#### METHOD

The soaps<sup>2</sup> used were sodium oleate, sodium myristate, sodium palmitate, sodium stearate, potassium palmitate and potassium stearate. Stock solutions contained 10 per cent pure saponified

<sup>2</sup> The soaps which were obtained from Sharpe and Dohme of Baltimore were prepared by saponifying 10 per cent of the pure fatty acids. These were adjusted to a pH of 9.0. Throughout this paper the terms "per cent sodium oleate," "per cent sodium myristate," etc., refer to the percentage of fatty acid present which has been saponified.

fatty acid. Dilutions were made from these stock solutions with sterile distilled water. The disinfectants used were phenol, meta-cresol, secondary butyl phenol, n-butyl resorcinol and

TABLE 1

*Effect of Na oleate on bactericidal activity of phenol, meta-cresol and butyl phenol at 37°C. against B. typhosus*

PER CENT Na OLEATE	PHENOL			META-CRESOL			SECONDARY BUTYL PHENOL		
	Dilution	Subcultures		Dilution	Subcultures		Dilution	Subcultures	
		1 minute	2 min- utes		1 minute	2 min- utes		1 minute	2 min- utes
5.0	100	+	-	100	+	-	100	+	-
5.0	150	+	+	150	+	-	150	+	-
5.0	200	+	+	200	+	+	200	+	+
4.0	100	-	-	100	-	-	100	+	-
4.0	200	+	+	200	+	+	200	+	+
3.0	100	-	-	100	-	-	100	+	-
3.0	200	+	+	200	+	-	200	+	+
2.0	100	-	-	100	-	-	100	+	-
2.0	150	+	-	150	-	-			
2.0	200	+	+	200	+	-	200	+	+
1.0	100	-	-	100	-	-	100	-	-
1.0	150	+	-	150	-	-	150	+	-
1.0	200	+	+	200	+	-	200	+	+
0.5	100	-	-	100	-	-	100	-	-
0.5	150	+	-	150	-	-	200	+	-
0.5	200	+	+	200	-	-	400	+	+
0.2							100	-	-
0.2							200	-	-
0.2							250	-	-
0.2							400	-	-
Controls									
5.0	0	+	+	0	+	+	0	+	+
0	100	-	-	200	-	-	2,000	-	-
NaCl control	0	+	+	0	+	+	0	+	+

+ growth on plates. - no growth on plates.

n-hexyl resorcinol. The organisms employed were *Bacillus typhosus* (Hopkins strain) and *Staphylococcus aureus* (from urinary infection), grown in plain broth (1.0 per cent Armour's peptone, 0.3 per cent Liebig beef extract and 0.5 per cent NaCl with a

pH of 6.8). Mixtures of soap and germicide in 5 cc. portions were placed in test tubes in a water bath at 37°C., to which was added 0.1 cc. of a twenty-four-hour broth culture of the test

TABLE 2

*Effect of Na oleate on bactericidal activity of butyl resorcinol and hexyl resorcinol at 37°C. against B. typhosus and Staph. aureus*

PER CENT Na OLEATE	DILU- TION BUTYL RESOR- CINOL	SUBCULTURES				DILU- TION HEXYL RESOR- CINOL	SUBCULTURES			
		<i>B. typhosus</i>		<i>Staph. aureus</i>			<i>B. typhosus</i>		<i>Staph. aureus</i>	
		1 min- ute	2 min- utes	1 min- ute	2 min- utes		1 min- ute	2 min- utes	1 min- ute	2 min- utes
5.0	100	+	-	+	-	100	-	-	-	-
5.0	200	+	+	+	+	200	+	+	+	+
5.0	250	+	+	+	+	250	+	+	+	+
5.0	500	+	+	+	+	500	+	+	+	+
5.0	1,000	+	+	+	+	1,000	+	+	+	+
4.0	100	-	-	-	-	100	-	-	-	-
4.0	200	+	+	+	-	200	+	+	-	-
3.0	100	-	-	-	-	100	-	-	-	-
3.0	200	+	+	+	-	200	+	+	-	-
3.0	1,000	+	+	+	+	1,000	+	+	+	+
2.0	100	-	-	-	-	100	-	-	-	-
2.0	200	+	-	-	-	200	-	-	-	-
2.0	1,000	+	+	+	+	1,000	+	+	+	+
1.0	200	-	-	-	-	200	-	-	-	-
1.0	400	-	-	-	-	400	-	-	-	-
1.0	500	+	+	+	-	1,000	+	+	+	+
0.5	200	-	-	-	-	200	-	-	-	-
0.5	500	-	-	-	-	500	-	-	-	-
0.5	700	+	+	+	-	1,000	+	-	+	-
0.2	500	-	-	-	-	200	-	-	-	-
0.2	1,000	-	-	-	-	1,000	-	-	-	-
0.2	1,500	+	-	+	-	1,500	-	-	-	-
Controls										
5.0	0	+	+	+	+		+	+	+	+
0	2,000	-	-	-	-	5,000	-	-	-	-

+ growth on plates. - no growth on plates.

organism. Subcultures to infusion agar pour plates were made after one and two minute intervals of exposure, incubated at 37°C. for forty-eight hours, and examined for growth.

THE EFFECT OF SODIUM OLEATE AND SODIUM MYRISTATE ON THE GERMICIDAL ACTIVITY OF PHENOL, META-CRESOL, SECONDARY BUTYL PHENOL, BUTYL RESORCINOL AND HEXYL RESORCINOL

Sodium oleate has a marked inhibitory effect on the germicidal activity of phenolic compounds (tables 1 and 2). A 5 per cent solution of this soap inhibits the action of 1:100 dilutions of phenol, meta-cresol, butyl phenol and butyl resorcinol against

TABLE 3

*Effect of 1 per cent Na myristate on the bactericidal activity of meta-cresol, butyl phenol and butyl resorcinol at 57°C. against B. typhosus*

META-CRESOL			SECONDARY BUTYL PHENOL			BUTYL RESORCINOL		
Dilution	Subcultures		Dilution	Subcultures		Dilution	Subcultures	
	1 minute	2 minutes		1 minute	2 minutes		1 minute	2 minutes
100	—	—	100	—	—	100	—	—
150	—	—	150	—	—	150	—	—
200	+	—	200	—	—	200	—	—
250	+	+	300	—	—	300	—	—
			400	—	—	400	—	—
			500	—	—	500	+	—
			600	+	—	600	+	+
			700	+	—	700	+	+
			800	+	+	800	+	+
			900	+	+	900	+	+
			1,000	+	+	1,000	+	+
Controls with no soap 200	—	—	2,000	—	—	2,000	—	—
1 per cent Na myristate							+	+

+ growth on plates. — no growth on plates.

*Bacillus typhosus* in one minute. After two minutes the organisms are killed. The same concentration of soap prevents the activity of 1:200 dilutions of these four compounds as well as of hexyl resorcinol after two minutes exposure. A 4 per cent solution of the soap gives the same results. As the concentration of the soap is decreased, this inhibitory action remains present, provided the amount of the disinfectant is likewise decreased.



A 0.5 per cent solution of sodium oleate is sufficient to destroy the bactericidal activity of so powerful a disinfectant as hexyl resorcinol in a 1:1000 dilution. This amount of the disinfectant is at least five times as much as is required to kill the organisms in aqueous solution in the same length of time. Frobisher (1927)

TABLE 4

*Effect of 1 per cent K. palmitate on bactericidal activity of meta-cresol, butyl phenol, butyl resorcinol and hexyl resorcinol at 37°C. against B. typhosus*

META-CRESOL			SECONDARY BUTYL PHENOL			BUTYL RESORCINOL			HEXYL RESORCINOL		
Dilution	Subcultures		Dilution	Sub-cultures		Dilution	Sub-cultures		Dilution	Sub-cultures	
	1 min-ute	2 min-utes		1 min-ute	2 min-utes		1 min-ute	2 min-utes		1 min-ute	2 min-utes
100	-	-	100	-	-	100	-	-	100	-	-
150	-	-	150	-	-	150	-	-	150	-	-
200	+	-	200	-	-	200	-	-	200	-	-
300	+	+	300	-	-	300	-	-	300	-	-
			400	+	-	400	-	-	400	-	-
			500	+	+	500	+	-	500	+	-
			600	+	+	600	+	-	600	+	-
			700	+	+	700	+	+	700	+	-
			800	+	+	800	+	+	800	+	-
			900	+	+	900	+	+	900	+	-
			1,000	+	+	1,000	+	+	1,000	+	-
									1,200	+	+
									1,400	+	+
Controls with no soap											
200	-	-	2,000	-	-	2,000	-	-	5,000	-	-
1 per cent K. palmitate control				+	+					+	+

+ growth on plates. - no growth on plates.

has reported that small amounts of sodium oleate enhance the activity of disinfectants by virtue of the ability of the soap to lower surface tension, thereby causing a more rapid destruction of the bacteria. In repeated experiments in which sodium oleate was used in our work, there was a suggestion that this might be true for phenol but not for the other disinfectants. Concen-

trations of less than 0.5 per cent sodium oleate seemed to have no effect whatsoever on the bactericidal action of the higher phenols even in very weak dilutions of the disinfectants.

Sodium myristate is slightly germicidal for *Bacillus typhosus* in a 5 per cent concentration. For that reason 1 per cent solutions were employed. In this concentration, its inhibitory action

TABLE 5

*Effect of 1 per cent K stearate on bactericidal activity of meta-cresol, butyl phenol, butyl resorcinol and hexyl resorcinol at 37°C. against B. typhosus*

META-CRESOL			SECONDARY BUTYL PHENOL			BUTYL RESORCINOL			HEXYL RESORCINOL		
Dilution	Subcultures		Dilution	Subcultures		Dilution	Subcultures		Dilution	Subcultures	
	1 min-ute	2 min-utes		1 min-ute	2 min-utes		1 min-ute	2 min-utes		1 min-ute	2 min-utes
100	—	—	100	—	—	100	—	—	100	—	—
150	+	—	150	—	—	150	—	—	150	—	—
200	+	—	200	+	—	200	—	—	200	—	—
300	+	+	300	+	—	300	—	—	300	—	—
			400	+	+	400	+	—	400	+	—
			500	+	+	500	+	—	500	+	—
			600	+	+	600	+	+	600	+	—
			700	+	+	700	+	+	700	+	+
			800	+	+	800	+	+	800	+	+
			900	+	+	900	+	+	900	+	+
			1,000	+	+	1,000	+	+	1,000	+	+
Controls with no soap											
200	—	—	2,000	—	—	2,000	—	—	5,000	—	—
1 per cent K stearate control										+	+

+ growth on plates. — no growth on plates.

on the sterilizing power of meta-cresol, butyl phenol and butyl resorcinol is very marked (table 3). Butyl phenol and butyl resorcinol are reduced in activity at least 75 per cent. Meta-cresol is less affected. A comparison with 1 per cent sodium oleate indicates that the germicidal activity of the compounds is reduced less by the addition of sodium myristate than by that of sodium oleate.

#### EFFECT OF POTASSIUM PALMITATE AND POTASSIUM STEARATE ON THE GERMICIDAL ACTIVITY OF CERTAIN PHENOLS

The potassium soaps of palmitic and stearic acids were used because of the high gelation properties of the two sodium soaps of the two acids. One per cent solutions were tested, and the same general inhibitory action displayed by sodium myristate and sodium oleate was shown to be present (tables 4 and 5). Potassium palmitate decreases the action of butyl phenol, butyl resorcinol and hexyl resorcinol at least 80 per cent. Potassium stearate acts as an even more inhibitory agent. When these results are compared with those for sodium myristate, it is apparent that as the fatty acid series is ascended, the soaps of the higher acids become progressively more inhibitory, *provided they are tested in the liquid or disperse phase*. This point will be taken up later in detail. When potassium stearate is compared with the corresponding soap of the oleic acid series, sodium oleate, there is a negligible difference in the inhibitory activity of the two soaps. This would seem to indicate that the amount of saturation of the fatty acid has no marked influence on the degree of inhibition exerted by the soap.

#### INFLUENCE OF PHYSICAL CONDITION OF PURE SOAPS ON THE BACTERICIDAL ACTION OF PHENOLS

Early in the work, the sodium soaps of palmitic and stearic acids were tested in 0.5 per cent solutions with varying concentrations of the disinfectants. The results were most irregular, but on the whole appeared to indicate that these two soaps had less influence on the bactericidal action of the germicides than sodium myristate or sodium oleate. No reason for this was apparent until the question of the physical condition of the soaps was taken into consideration. Solutions of these soaps of 0.5 per cent concentration form soft gels very quickly, even at 37°C. In making up a series of test dilutions it was necessary to melt the stock solutions and measure out the quantity desired. By the time the soap was mixed with varying concentrations of the germicide and the test made, some of the final mixtures were

geled. This often gave results which made it appear that the soap interfered with large amounts of the disinfectants to a greater extent than with smaller amounts. It occurred to us that this might be due entirely to the physical condition of the soap, and experiments were devised to determine this point. When the tests were run before the soap had geled there was marked interference with the disinfectant. When run after a gel had formed, there was less interference (see table 6). The extreme signifi-

TABLE 6  
*Influence of physical condition of Na palmitate on germicidal properties of butyl resorcinol, 37°C.*

PER CENT Na PALMITATE	DILUTION OF BUTYL RESORCINOL	A*		B†	
		<i>B. typhosus</i>		<i>B. typhosus</i>	
		1 minute	2 minutes	1 minute	2 minutes
0.5	700	—	—	—	—
0.5	800	++++	+	—	—
0.5	900	++++	+	—	—
0.5	1,000	++++	+	—	—
0.5	1,200	++++	++	—	—
0.5	1,400	++++	++	—	—
0.5	1,600	++++	+++	—	—
0.5	1,800	++++	++++	—	—

+ = growth. — = no growth.

\* A: Butyl resorcinol kept at 37°C. Na palmitate added. Tests run before gel formed.

† B: Butyl resorcinol and Na palmitate mixed. Allowed to set for two hours at room temperature. Temperature raised to 37°C. and tests run.

cance of this is realized when one considers that the detergent properties of soaps parallel the amount of colloidal soap present (McBain, 1920).

#### DISCUSSION

The data in the foregoing pages bring up for consideration points of both theoretical and practical importance. Although no satisfactory and complete explanation for the effect of soaps on the germicidal properties of phenols can be offered from a study based solely on germicidal action of the disinfectants,

the results stimulate speculation. Four different possibilities present themselves:

1. The occurrence of a true chemical reaction between the soap and the phenolic compound, resulting in a non-germicidal substance.

2. A protective colloidal action on the part of the soap for the bacteria as suggested by Frobisher (1927).

3. The ability of the soap to remove the phenolic substance from the solution in a definite partition-coefficient ratio.

4. A combination of physico-chemical factors which may or may not have been expressed in the first three statements.

It would be a difficult matter to present any evidence to support the first statement in a paper of this type with results based on strictly bacteriological methods. However, in a long series of detailed experiments, which are not tabulated in this publication, an effort was made to show a definite molecular relationship between the amount of soap required to inactivate the phenolic substance and the amount of germicide present. The destruction of viability of the bacteria was used as an index of the presence of free germicide. As might be expected from a technic so unsuitable for this type of determination, the results were irregular and indefinite in their conclusions.

The theory of Frobisher offers an interesting and simple explanation. The protective colloidal action of soaps has been amply demonstrated by Papaconstantinou (1925) who assigned definite "gold numbers" to different pure soaps. If this idea is applied directly to the case of soaps and phenolic substances, we would pre-suppose a suspension of soap-in-water and phenolic compound-in-water, to which would be added the bacterial culture. The bacteria would be immediately surrounded by a film of soap and hence protected from the action of the germicide. This theory might be easily evolved from working with phenols only in true solutions. However, when one handles super-saturated solutions of these compounds, other facts are observed which render so simple an explanation untenable. For instance, 1 part of butyl phenol added to 99 parts of water is not a true solution, but may be shaken into a fine suspension of oil globules

in water. If one drop of 10 per cent sodium oleate is added to 1 cc. of this mixture, the oil globules immediately disappear, and the solution becomes clear. The same holds true for emulsions of butyl resorcinol and hexyl resorcinol. This can not be due to the free alkali present in the soap, since it requires less sodium oleate to obtain a water clear solution of the germicide than sodium carbonate or sodium hydroxide. Furthermore, when solutions containing 1 per cent butyl phenol and 5, 4, 3, 2, 1 and 0.5 per cent sodium oleate, respectively, are tested for germicidal activity, the first four solutions (containing the higher concentration of soap) are inactive in one minute against *Bacillus typhosus*. The remaining two are germicidal. In the last tube, globules of oil are plainly visible, indicating a supersaturation of butyl phenol both for the soap and for the water. When the experiment is repeated with 0.5 per cent butyl phenol, the general results are the same. The germicidal action of the disinfectant is evident in a concentration of soap slightly more than is necessary to hold the butyl phenol in solution.

The condition seems analogous to that of the well known phenol-alcohol-water combination in which Reichel (1909) has shown that although phenol in alcohol is non-germicidal, by decreasing the amount of alcohol or by adding NaCl the partition-coefficient ratio of the phenol between the alcohol and the water is increased for the water, and therefore more phenol is in solution and the bacteria are killed. Efforts to increase the water solubility of at least one of the compounds, hexyl resorcinol, in the presence of soap by adding sodium carbonate, sodium hydroxide and sodium chloride were unsuccessful. The recent work of Cooper and Sanders (1927) with sodium stearate and phenol lends support to this hypothesis. These workers find a definite partition-coefficient ratio between the concentration of phenol in water and in the soap. By increasing the amount of phenol, the increased phenol uptake with sodium stearate is associated with passage of the soap into solution.

It seems unlikely, however, that the actual explanation of the inhibitory effect of soaps on the bactericidal activity of phenols is fully accounted for by either, or both, the second and third

hypotheses, since there are other factors which should be taken into consideration. The very fact that soaps are colloidal electrolytes must necessarily complicate any simple explanation. The problem is one of theoretical importance and interest, and our results suggest that its final solution lies in the hands of the physical chemist.

From a practical standpoint, this work indicates the unsuitability of phenolic compounds as germicidal agents in soaps. A germicidal soap is expected to perform a double function. It must cleanse the surface, whether this surface be the skin, floors, or walls, etc. In skin disinfection, it should be expected to destroy whatever bacteria are mechanically washed off in the lather, as well as those remaining on the skin; at the same time it should be capable of some penetration into the depths of surface irregularities. In this discussion no consideration will be given to the question of penetration.

Our experimental results indicate that the only possibility of producing a germicidal soap with a phenolic compound, which will destroy the bacteria mechanically removed from the skin, lies in the addition of an excess of the disinfectant. The use of the lower or cruder phenols is ruled out because this class of substances is not only poisonous but extremely irritating to the skin in the concentrations which would be necessary to destroy bacteria. Theoretically, it is possible to use a non-irritating substance such as hexyl resorcinol in large enough quantities to yield a germicidal soap, but from a practical standpoint the cost of such a product precludes its general use at present. The fact that the disinfectant action of phenol is interfered with to such a pronounced extent by the disperse phase of the soap must also be taken into account. For example, a cake of soap containing as much as 5 per cent of a phenolic compound of high bactericidal efficiency would not be bactericidal when used for washing the hands. McBain, cited by Fall (1927), has listed seven factors which are necessary for detergent action. Among these factors are the necessity of having the soap in solution and the necessity of having the soap in colloidal form. It should be emphasized that this colloidal or disperse phase has been

shown to be more highly inhibitive to the germicidal action of phenols than the soft gel phase. Obviously, as the soap is diluted, the germicide is not only diluted, but at the same time its activity is interfered with more extensively. This fact alone also eliminates the practicability of obtaining a liquid soap with germicidal properties which could be attributed directly to a phenolic compound incorporated with it.

### CONCLUSIONS

1. The marked inhibitory action of sodium oleate, sodium myristate, potassium palmitate and potassium stearate on the bactericidal properties of phenol, meta-cresol, secondary butyl phenol, n-butyl resorcinol and n-hexyl resorcinol has been demonstrated. The relationship appears to be more or less quantitative and indicates the impossibility of producing a germicidal soap by adding small quantities of a phenolic compound to soap. The addition of large quantities of a phenolic disinfectant is not feasible, since the cost of production precludes the use of any but very crude and irritating phenols. Phenols are therefore unsuitable as disinfectant agents in the production of germicidal soaps.

2. The germicidal activity of a phenolic compound is interfered with more extensively by the disperse phase than by the gel phase of soaps.

3. Several theories to explain the inhibitive action of soaps on the bactericidal activity of phenols have been discussed. Evidence has been introduced to show that the soap removes the phenolic substance from the solution and thereby interferes with its bactericidal activity, since this activity is dependent upon the solution of the phenol in water.

### REFERENCES

- COOPER, E. A., AND SANDERS, E. 1927 Relations of phenol to proteins and other colloids. *Jour. Phys. Chem.*, **31**, 1-22.
- EGGERTH, A. H. 1927 The effect of pH on the germicidal action of soaps. *Jour. Gen. Phys.*, **10**, 147-160.
- FALL, P. H. 1927 Detergent action of soaps. *Jour. Phys. Chem.*, **31**, 809-849.
- FROBISHER, MARTIN, JR. 1927 Studies upon the relationship between surface tension and the action of disinfectants, with special reference to hexyl-resorcinol. *Jour. Bacteriol.*, **13**, 163-182.



- HAMILTON, H. C. 1917 Facts and fallacies of disinfection. Amer. Jour. Pub. Health, **7**, 282-295.
- McBAIN, J. W. 1920 Third Report on Colloid Chem. Brit. Assoc. Adv. Science.
- PAPACONSTANTINOU, B. 1925 Protective action of soaps on Zsigmondy's gold solutions. Jour. Phys. Chem., **29**, 319-322.
- REICHEL, H. 1909 Zur Theorie der Desinfektion. Biochem. Ztschr., **22**, 149-200.
- RETTGER, L. F., VALLEY, G. AND PLASTRIDGE, W. N. 1928 Studies on the disinfectant properties of certain alkyl phenols. Centralblatt f. Bakteriologie, (in press).
- TILLEY, F. W., AND SCHAEFFER, J. M. 1927 Germicidal efficiency of cocoanut oil and linseed oil soaps and of their mixtures with cresol. Jour. Infec. Dis., **37**, 358-367.
- WALKER, J. E. 1924 The germicidal properties of chemically pure soaps. Jour. Infec. Dis., **35**, 557-566.
- WALKER, J. E. 1925 The germicidal properties of soap. Jour. Infec. Dis., **37**, 181-192.
- WALKER, J. E. 1926 The germicidal properties of soap. Jour. Infec. Dis., **38**, 127-130.

# A STUDY OF MICROCOCCUS ZYMOGENES<sup>1</sup>

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In 1899, MacCallum and Hastings isolated from the blood and organs of a person dying of acute endocarditis an organism which they described and named *Micrococcus zymogenes*. It produced tiny, smooth, glistening, white colonies on agar plate cultures. Morphologically the organism was found to be a minute coccus, often somewhat elongated, occurring in masses, singly, and in pairs or short chains of pairs. It was non-motile, non-encapsulated and Gram-positive. It grew well in glycerol- or ascitic-fluid-agar and other nutrient media. Glucose and lactose were fermented with the formation of acid but no gas. Litmus was reduced. A thin, dirty white, pasty growth appeared irregularly on potato. No indol was formed in nutrient bouillon. The organism was a facultative anaerobe and resisted drying on agar slants for months.

The proteolytic power of the organism was one of its most conspicuous properties. Gelatine was liquified, coagulated serum was digested, and milk was first coagulated and then digested. The coagulation was thought to be due to a rennin-like enzyme, in part, at least. The changes produced in litmus milk were regarded as unusual and very characteristic. Sterile filtrates of broth cultures produced proteolysis similar to, but not so extensive as, that caused by the live organisms themselves.

The micrococcus was found to be pathogenic for rabbits and white mice. Intraperitoneal injections of 0.3 to 0.7 cc. of broth

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suspensions resulted in the death of mice in from seven hours to four days. No exo-toxin was demonstrated.

An organism which had previously been isolated by Harris from an old cesspool was believed, by these authors, to be identical with *M. zymogenes*. After long cultivation on agar the latter organism was found to be non-pathogenic for mice.

Harris and Longcope, in 1900, reported finding *M. zymogenes* in four autopsies, the diseases being tuberculosis, cystitis, nephritis and gastric carcinoma.

In 1905 Birge reported finding, in the laryngeal mucous exudate of certain crows, an organism culturally and immunologically identical with that of MacCallum and Hastings but less pathogenic for rabbits.

*M. zymogenes* was mentioned for the first time in British bacteriology by Hicks in 1912. His organism differed from that of MacCallum and Hastings in being non-pathogenic for mice and in failing to digest coagulated serum. Pathogenicity for mice is seen to be a somewhat variable factor, but the failure to digest serum raises the question as to whether Hicks was dealing with a strain of *M. zymogenes* or with some one of the less proteolytic but closely related streptococci.

Crowe, in 1923, mentioned the occurrence of *M. zymogenes* in a case of malignant endocarditis. He also reports finding it frequently in the intestinal tract of normal persons (1928).

It is the purpose of this paper to report the isolation, from new sources, of five strains of organisms corresponding to the original description of *M. zymogenes* and to describe additional characters of the organism, as well as its relation to certain other species which it closely resembles. It is desired also to point out the value of making, routinely, studies of the proteolytic as well as the fermentative powers of all strains of streptococcus-like bacteria.

#### SOURCES OF CULTURES

Of over 200 cultures of streptococci isolated during a bacteriologic study of the throats, sinuses, etc., in cases of acute and subacute glomerular nephritis by Longcope, O'Brien,

McGuire, Hansen and Denny (1927), two from the tonsils were found to digest coagulated pork serum (Loeffler's medium) and to liquefy gelatin. At about the same time, a bacteriologic study of the genital tract of a large number of women in the Johns Hopkins obstetrical dispensary was being made by Harris, Curran and Brown (1928). From the normal cervix in one case and from the normal vagina in another, streptococcus-like organisms were isolated which were found to liquefy gelatin and digest coagulated pork serum. These were very kindly given to us by Dr. Harold R. Curran who isolated them. A fifth culture, labeled *M. zymogenes*, was kindly sent to us by Dr. H. Warren Crowe of the Pickett-Thompson laboratory in London. Streptococcus no. 799 (*S. liquefaciens*) was purchased from the American type culture collection at Chicago. These six cultures were subjected to study by the methods to be described.

#### FERMENTATION REACTIONS

To 5 cc. of sugar-free infusion bouillon there were added two drops of sterile horse serum and 0.5 cc. of a 10 per cent sterile, aqueous solution of the desired test substance. Inoculation was made with fresh broth growth of the organism and the culture was incubated five days aerobically at 37°C. At the end of this time the pH of the culture was determined by the method of Brown (1924).

#### PROTEOLYSIS

Loeffler's slants, infusion gelatin tubes, tubes of cooked meat medium under vaseline, tubes of litmus milk and tubes containing 5 cc. of broth with a small cube of egg-white were inoculated and incubated as above. At the end of ten days the gelatin tubes were placed for three hours in a jar of cold water in the refrigerator at about 5°C. The level of the ground meat in the tubes of cooked meat medium was carefully marked before inoculation and subsidence noted at the end of ten days. Coagulation and digestion of the milk and digestion of the Loeffler's medium and egg-white were noted as they proceeded. To determine the extent of proteolysis, formol titrations were made on

all of the above cultures to determine increase in formol titrable bodies. The method "B" of Brown (1923) was used. It should be pointed out that this method includes ammonia in its titration as well as amino acids. Ammonia may result from the decomposition of polypeptids, etc., and not from proteolysis. However, in this case, proteolysis was perfectly visible since the material actually disappeared and became fluid. Furthermore, as Orla-Jensen (1919) has shown, the lactic acid streptococci (to which *M. pyogenes* is undoubtedly closely related) do not split amino acids. For this reason no ammonia determinations were made. Where proteolysis was visible, marked increase in formol-titrable bodies occurred.

As controls on the formol titrations of cooked meat medium, two strains of *Streptococcus pyogenes*, one a histase producer and the other a non-histase producer (Frobisher, 1926) were inoculated into similar media at the same time. Neither of these strains is known to have any proteolytic effect on milk, gelatin, coagulated serum or egg-white.<sup>3</sup>

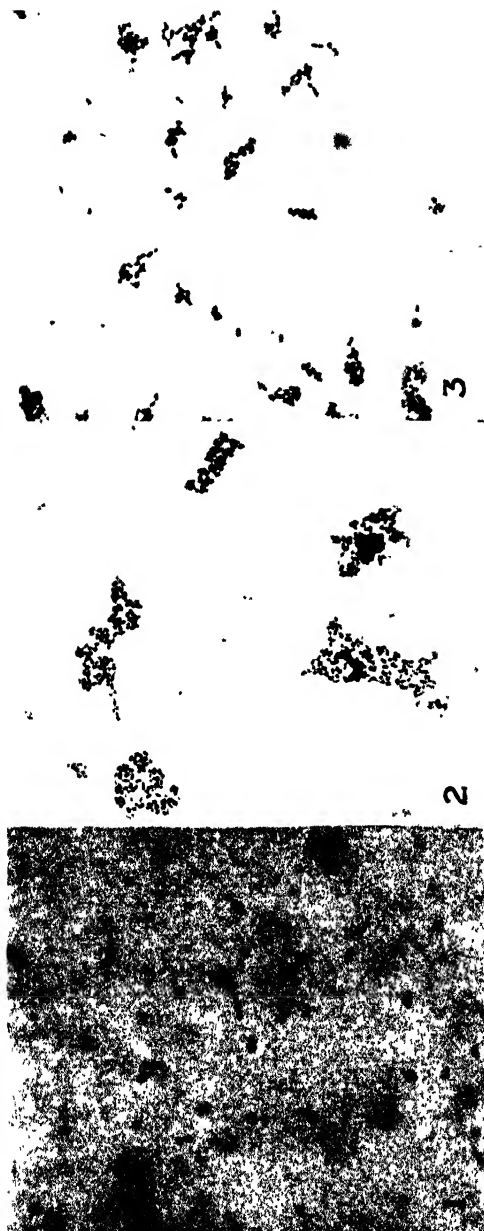
#### HYDROLYSIS OF SODIUM HIPPURATE

Infusion bouillon containing 1 per cent of sodium hippurate was used. At the end of ten days incubation at 37°C. a qualitative test for benzoic acid was made with ferric chloride in HCl solution according to the method of the originators of the test (Ayers and Rupp, 1922). This test was supplemented by a quantitative determination of glyocol using the formol titration method referred to above. As controls on the titration, two streptococci, one a known hydrolyzer and one a known non-hydrolyzer, were included. Control titrations were also made of cultures of all the organisms in hippurate-free broth and in sterile hippurate broth.

#### ACTION ON BLOOD

For each blood agar pour plate, 1 cc. of defibrinated rabbit's blood was added to about 12 cc. of melted agar, cooled to about

<sup>3</sup> In making formol titrations on Loeffler's slants, 2 cc. of distilled water were added to the water of condensation and the tubes allowed to stand for about three hours before making the titration.



FIGS. 1 TO 3

FIG. 1. *Micrococcus zymogenes*. Broth culture of strain AI. ( $\times 1000$ )  
FIG. 2. *Micrococcus zymogenes*. Broth culture of strain "C". ( $\times 1000$ .)  
FIG. 3. *Micrococcus zymogenes*. Broth culture of strain F33. ( $\times 1000$ )

TABLE 1  
*Biochemical characters of M. zymogenes, etc.*

STRAIN NUMBER	SOURCE	DATE ISOLATED	DATE STUDIED	CONDITIONS OF STORAGE DURING TIME ISOLATED AND DATE STUDIED	APPEARANCE OF COLONIES ON AGAR	MORPHOLOGY (IN BROTH)	GRAM STAIN	MOTILITY (CAPILLARIES)	ACTION ON LITMUS MILK	DIGESTION OF SERUM (LOEFLER)
A1	Tonsil—case of nephritis	December 7, 1926	January 28, 1928	Agar slants. Monthly renews till May, 1927. Dried in vacuo. Taken from vacuum to broth (Vacuum—see Brown, 1925)	Up to 1 mm in diameter. Whitish, round, gluing and opaque	Small, ovoid cocci in masses, pairs and short chains	Retained	—	According to original description—typical. Coagulation, digestion, curd gone 15 days. Red residue	+
A2	Same as above	December 7, 1926	January 28, 1928	Same as above	Same as above	Same as above	Retained	—	Same as above	+
F63	Normal vagina	January 20, 1928	January 28, 1928	Agar slant	Same as above	Same as above	Retained	—	Same as above	+
F33	Normal cervix	December 13, 1927	January 28, 1928	Dried in vacuo (Brown, 1925)	Same as above	Same as above	Retained	—	Same as above	+
Crowe	Feces	Not stated	April, 1928	Not stated	Same as above	Same as above	Retained	—	Same as above	+
799 ( <i>S. liquefaciens</i> )	Milk	Not stated	June, 1928	Not stated	Same as above	Same as above	Retained	—	Same as above	+

STRAIN NUMBER	HISTAB	DIGESTION OF EGG-WHITE	TYPE OF COLONIES IN BLOOD AGAR	GROWTH ON POTATO	FERMENTATION* OF								HYDROLYSIS OF SO-DIUM HIPPOPHOSPHATE	SOLUBLE HEMOLYMIN IN BROTH	INDOL PRODUCTION	NITRATE REDUCTION	SURVIVAL OF 65°C.	EFFECT ON ANIMALS
					Lactose	Mannitol	Saltin	Glucose	Raffinose	Sucrose	Starch	Inulin	Glycerol					
A1	+	-	Beta	Thin, whitish	+	+	+	+ pH 4.6	-	+	-	-	+	+	-	-	-	1 cc.; mouse dead 18 hours 3 cc.; rabbit dead 7 days; mitral vegetations
A2	+	-	Beta	As above	+	+	+	+ pH 4.5	-	+	-	-	+	+	-	-	-	1 cc.; mouse very sick; survived 3 cc.; rabbit sick; survived
F63	+	-	Beta	As above	+	+	+	+ pH 4.8	-	+	-	-	+	+	-	-	-	Same as above Same as above
F33	+	-	Alpha	None	+	+	+	+ pH 4.6	-	+	-	-	+	+	-	-	-	Same as above Same as above
Crowe	+	-	Gamma	None	+	+	+	+ pH ...	-	+	-	-	+	+	-	-	-	1 cc.; mouse not affected Rabbit not injected
799	+	-	Gamma	Little if any	+	+	+	+ pH 4.6	-	+	-	-	+	+	-	-	-	1 cc.; mouse not affected Rabbit not injected

\* + = acid.



42°C. This was inoculated with a broth dilution of a young culture so as to give from 50 to 150 colonies per plate. The mixture was poured into a petri dish and incubated aerobically for twenty-four hours at 37°C. At the end of this time *deep* colonies were carefully examined with the low power microscope to determine whether they were of the alpha or beta types of Smith and Brown (1915), or the gamma type of Brown (1919). These determinations were supplemented by test tube hemolysin tests, using eight-hour-old, well grown, 20 per cent horse-serum broth cultures as described by de Kruif and Ireland (1920).

#### IMMUNOLOGICAL STUDIES

Rabbits were immunized against strains A2, 33 and 63. The sera were tested against strains A1, A2, 33 and 63. The agglutination tests show that the alpha and beta types are closely related immunologically, but that they can, by dilution, be differentiated into immunological types. Neither the gamma strain nor *S. liquefaciens* has as yet been studied in this respect.

#### ANIMAL PATHOGENICITY

All of the animals were inoculated with fresh cultures made from stock which had been kept for some time under artificial conditions. Variations in pathogenicity are so common that differences in effect on animals among these strains is not regarded as of any special significance. Mice were inoculated intraperitoneally with 1 cc. of eighteen-hour-old infusion broth cultures. Rabbits were inoculated intravenously with several successive doses in amounts varying from 1 to 3 cc.

#### CAPSULES, STAINING, MORPHOLOGY, APPEARANCE OF GROWTH ON AGAR

*Determination of capsules* was made with nine-hour-old cultures on fresh blood agar slants as follows: A loopful of sterile broth was placed on a clean slide. Some of the growth from the blood agar slant was emulsified in this and a loopful of India ink mixed with the emulsion. A cover slip was immediately placed

upon the mixture and the bacteria examined with the oil immersion lens.

*Staining reactions, morphology and appearance on various media* were studied during the procedures outlined above. Figures 1 and 3 show the microscopic appearance of strains number A1 and F33 respectively, in broth culture. Dr. Crowe's strain is shown in figure 2.

#### RESISTANCE TO DRYING AND OTHER CONDITIONS

Table 1 shows the date of isolation of the cultures and the conditions under which they have been kept since. All are still alive.

#### OTHER TESTS

The power of *nitrate reduction* was determined by aerobic incubation at 37°C. for five days, of cultures in 1 per cent peptone solutions containing 0.1 per cent sodium nitrate. The presence of nitrite was tested for by adding successively 6 drops of sulphanilic acid solution and an equal amount of dimethyl-alpha-naphthalamine solution (Wallace and Neave, 1927).

The presence of *indol* was tested for in 1 per cent peptone solution after ten days incubation by adding Boehm's reagent (acid solution of paradimethylamidobenzaldehyde).

*Heat resistance* was determined by placing 5 cc. of twenty-four-hour broth cultures (in cotton stoppered, 12 mm. tubes) in a deep water bath at 65°C. and making subcultures after various intervals.

#### RESULTS

The results of all the tests and determinations described above are shown in table 1.

#### DISCUSSION

##### *A. Varieties of the organism*

It is seen in table 1 that, by applying the tests originally described by MacCallum and Hastings, the six organisms described

in this paper might all be classed as *M. zymogenes*. The fact that one of the strains produces colonies of the alpha type in blood agar plates while other strains produce colonies of the beta type or the gamma (or indifferent) type indicates that the organisms studied constitute in reality a group of related varieties.

A discussion of the systematic position and species identity of *M. zymogenes* is of interest at this juncture.

There are two groups of streptococci which have been studied in considerable detail but regarding the systematology of which there is still considerable discussion. These are the groups comprising the fecal streptococci and the lactic acid or milk streptococci. It seems quite probable that there is considerable overlapping of these groups since many of the milk streptococci are undoubtedly derived from intestinal sources. A large number of species have been described by students of each group and the literature is quite extensive. The monograph by Orla-Jensen (very kindly loaned by Dr. G. J. Hucker) and the paper by Dible (1921) are of interest in this connection. In general it may be said that the organisms of each group resemble one another in being Gram positive, more or less ovoid cocci, facultative aerobes, commonly forming pairs and short chains, possessing more or less resistance to temperatures around 60°C., growing well in milk over quite a wide range of temperatures and occurring chiefly in feces, stable dirt, milk and dairy products and utensils and in the alimentary tract. There are a few pathogens. Many, of course, depart quite widely from this generalized description. Details regarding various types may be found in the literature referred to above and elsewhere.

In a general way also it appears that there may be traced throughout the entire group a sort of metabolic gradient. At one extreme are organisms possessing slight metabolic powers, as for example, certain types of the *Enterococcus* such as that described by Macé which fermented nothing, or some few of the fecal streptococci described by Dible, fermenting mannitol only, not liquefying gelatin or peptonizing milk and having no pathogenic properties and not being heat resistant. At the other extreme are organisms like *S. glycerinaceous*, *S. lique-*

*faciens*, *S. apis* and possibly *M. zymogenes*, which attack a great variety of carbohydrates, liquefy gelatin, peptonize casein, produce odors and flavors in milk and cheese and are heat resistant.

Two questions arise in connection with *M. zymogenes*. First, is this organism identical with or merely closely related to *S. liquefaciens*? Second, if not identical with *S. liquefaciens*, should *M. zymogenes* be classed as a streptococcus? Freudenreich, (1894) originally described *S. liquefaciens*, called by him *M. casei-amari*, as an oval diplococcus producing tiny greyish colonies on agar, liquefying gelatin, peptonizing milk and producing bitter cheese and being resistant to 65°C. for ten minutes. Orla-Jensen describes the organism as a glycerol fermenting, gelatin liquefying, casein digesting oval diplococcus forming long chains, or short chains and pairs. According to this author the organism resists 70° to 75°C. Ford (1927) describes *S. liquefaciens* as a small oval coccus producing tiny whitish colonies on agar, fermenting lactose, glucose, and sucrose, producing scanty growth on potato, liquefying gelatin, and peptonizing milk. He does not mention digestion of serum, but describes growth on it.

By consulting table 1 it will be seen that *M. zymogenes* resembles *S. liquefaciens* in practically every particular. The strain of *S. liquefaciens* was no more resistant to heat than *M. zymogenes*. It appears entirely probable that *M. zymogenes* is simply a variety of *S. liquefaciens* if not actually identical with it, and in any case should be classed as a streptococcus.

### B. The enzymes

The proteolytic enzymes of these organisms and their relation to proteases produced by streptococci may be mentioned here. Frobisher (1926) described a tissue digesting enzyme (histase) produced by beta type hemolytic streptococci. Histase was thought to resemble trypsin in its action, but the streptococci which produced it had no demonstrable action on gelatin, coagulated serum or egg white. Whether the organisms described in this paper produce several types of proteolytic enzyme,

or only one enzyme, capable of wider powers of digestion than histase, cannot be stated at present.

### *C. Relation of proteolysis to hemolysis*

Julianelle (1922) demonstrated the existence of a relationship between proteolysis and hemolysin production by staphylococci. Frobisher's study of hemolysin and histase production by streptococci showed little if any connection to exist between these properties in the case of these organisms. Hemolysin production and proteolysis by members of the group described in this paper appear to be different functions, since the alpha type and gamma type strains are as proteolytic as the beta type strains. It is seen also that while broth cultures have no hemolytic effect upon 5 per cent rabbit cell suspensions, filtrates of broth cultures of *M. zymogenes*, as shown by MacCallum and Hastings, contain the active, proteolytic enzyme.

### *D. Relation to disease*

*M. zymogenes*-like organisms have been isolated from such a variety of sources as to raise some doubt of their relation to any specific type of pathological condition. *M. zymogenes* has been reported as isolated from cases of malignant endocarditis frequently enough to suggest the existence of a relationship to this condition. The experimental production of vegetations on the mitral valves in rabbits with *M. zymogenes* is not of special significance since this may be done with a variety of streptococci (Clawson, 1925; Birkhaug, 1927; Brown, 1928; and others). In the rabbits injected by us nothing resembling Aschoff bodies were observed although mitral vegetations were produced in the rabbit by strain A1.

### SUMMARY AND CONCLUSIONS

1. A review of the literature upon, and a biochemical study of, five newly isolated strains of *M. zymogenes* and one stock strain of *S. liquefaciens* has been made. New characters are described.
2. The organisms studied as *M. zymogenes* may not all belong

to a single species, since some produce alpha type, some beta type and some gamma type colonies in blood agar pour plates. A certain degree of differentiation on an immunological basis is possible.

3. The resemblance of the organisms studied as *M. zymogenes* to *S. liquefaciens* is such as to suggest that the former are merely varieties of the latter or that *M. zymogenes* and *S. liquefaciens* are identical. *M. zymogenes* should be classed as a streptococcus.

4. The proteolytic enzymes of these organisms resemble histase in their action on cooked meat but differ from this enzyme in their ability to digest coagulated serum, gelatin and casein as well.

5. There appears to be no relation between hemolysin and proteolytic enzyme production by these organisms.

6. The literature reveals nothing to suggest a direct relationship between *M. zymogenes* and any special type of pathological condition although organisms called *M. zymogenes* have been more frequently isolated from endocarditis than from any other single disease.

7. Proteolytic streptococci of the type represented by *S. liquefaciens* might be more frequently reported in pathological bacteriology if more detailed study of the proteolytic activity of streptococcus-like organisms were made as a routine.

During the preparation of the above paper the valuable work of Hucker (1928) dealing with the proteolytic cocci has appeared and the reader is referred to this for further discussion of these cocci and their relationship to *M. zymogenes*. We are indebted to Dr. Hucker for helpful suggestions in this connection.

#### REFERENCES

- AYERS, S. H., AND RUPP, P. 1922 Differentiation of hemolytic streptococci from human and bovine sources by the hydrolysis of sodium hippurate. *Jour. Inf. Dis.*, **30**, 388.
- BIRGE, E. G. 1905 Some observations on the occurrence of *Micrococcus zymogenes*. *Bull. Johns Hopkins Hosp.*, **16**, 309.
- BIRKHAUG, K. E. 1927 Rheumatic fever. *Jour. Inf. Dis.*, **40**, 549.
- BROWN, J. H. 1919 The use of blood agar for the study of streptococci. *Rockefeller Institute Monograph No. 9*.

- BROWN, J. H. 1923 The formol titration of bacteriological media. *Jour. Bact.*, **8**, 245.
- BROWN, J. H. 1924 The colorimetric determination of the hydrogen ion concentration of small amounts of fluid. *Jour. Lab. and Clin. Med.*, **9**, no. 4.
- BROWN, J. H. 1925 The preservation of bacteria in vacuo. *Abs. Bact.*, **9**, no. 1.
- BROWN, J. H. 1928 Personal communication.
- CLAWSON, B. J. 1925 The etiology of rheumatic fever. *Jour. Inf. Dis.*, **36**, 444.
- CROWE, H. W. 1923 A differential medium for streptococci. *Jour. Pathol. and Bact.*, **26**, 51.
- CROWE, H. W. 1928 Personal communication.
- DE KRUIF, P. H., AND IRELAND, P. M. 1920 Streptolysin. *Jour. Inf. Dis.*, **26**, 285.
- DIBLE, J. H. 1921 The enterococcus and the fecal streptococci. *Jour. Pathol.*, **24**, 3.
- FREUDENREICH, E. VON 1894 Beitrage zur Kenntniss der Ursachen des bittern Käses und der bittern Milch. *Landwirt. Jahrb. der Schweiz.*, **8**, 135.
- FORD, W. W. 1927 Textbook of Bacteriology. W. B. Saunders, Philadelphia, Pa.
- FROBISHER, M., JR. 1926 A tissue-digesting enzyme (histase) of streptococci. *Jour. Exp. Med.*, **44**, 777.
- HARRIS, N. M., AND LONGCOPE, W. T. 1901 *Centralb. f. Bakt.*, 1 Abt., **30**, 353.
- HARRIS, J. W., CURRAN, H. R., AND BROWN, J. H. Work in preparation (1928).
- HICKS, B. 1912 An unusual organism (*M. zymogenes*) in a case of malignant endocarditis. *Proc. Roy. Soc. Med. (Pathol. Sec.)*, **5**(3), 126.
- HUCKER, G. J. 1928 Studies on the Coccaceae, Technical Bull. No. 144. N. Y. State Agric. Exp. Sta.
- JULIANELLE, L. A. 1922 Studies of hemolytic staphylococci. *Jour. Inf. Dis.*, **31**, 256.
- LONGCOPE, W. T., O'BRIEN, D. P., MCGUIRE, J., HANSEN, O. C., AND DENNY, E. R. 1927 The relationship of acute infections to glomerular nephritis. *Jour. Clin. Invest.*, **5**, 7.
- ORLA-JENSEN, S. 1919 The Lactic Acid Bacteria. (Monograph.) Copenhagen.
- MACCALLUM, W. G., AND HASTINGS, T. W. 1899 A case of acute endocarditis caused by *Micrococcus zymogenes*, with a description of the organism. *Jour. Exp. Med.*, **4**, 521.
- MACÉ 1912 *Traite pratique de Bacteriologie*. Paris, Balliere et Fils. Cited from Dible (loc. cit.).
- SMITH, T., AND BROWN, J. H. 1915 A study of streptococci isolated from certain presumably milk-borne epidemics occurring in Massachusetts during 1913 and 1914. *Jour. Med. Res.*, **31**, 455.
- WALLACE, G. I., AND NEAVE, S. L. 1927 The nitrite test as applied to bacterial cultures. *Jour. Bact.*, **14**, 377.

# PHYSIOLOGICAL STUDIES OF CELLULOSE FERMENTATION

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## INTRODUCTION

The secrets of rapidity and efficiency in the process of cellulose fermentation seem to rest with nature. Man's attempts to study the phenomenon in the laboratory have been woefully ineffective. Very little is actually known concerning the agents involved, and as yet no method has been devised whereby they may be more thoroughly investigated while exercising, or capable of exercising, such cellulose-dissolving ability as they may possess in a natural habitat. The inevitable result of the artificial laboratory method has been inactivity and inefficiency on the part of the microorganism.

By studying the elemental needs of the cellulose destroyer, aided by suggestions from nature, it should be possible to obtain, under controlled conditions, an artificial fermentation of cellulose of maximum efficiency, even surpassing that commonly observed in nature.

The most important factors concerned in the artificial fermentation of cellulose seem to consist of the following: (a) Form of cellulose employed, (b) nitrogenous constituent of the medium, (c) provision of essential food or stimulating factors, (d) associative action of microorganisms. In previous papers (Sanborn, 1926, 1927) the author has shown that the growth and physiological efficiency of cellulose destroyers are markedly influenced by the presence of a so-called essential food factor in the medium and by microbial associations. By means of the China blue-aurin-cellulose medium (Sanborn 1927) it is possible to demonstrate the rôle of these stimulative factors very effectively.



## EXPERIMENTAL

In the previous work it was found desirable to employ a natural, unaltered form of cellulose. Raw cotton, which was selected, proved highly satisfactory. The seed fiber of the milkweed was also very useful, particularly in microscopic methods. Filter paper, in general, proved to be relatively resistant to attack.

In the preparation of the China blue-aurin-cellulose medium precipitated raw cotton was employed, also a nutrient solution

TABLE I  
Cellulose decomposition by soil fungi

	BLUE ZONE DEVELOPMENT					CLEAR ZONES APPEAR IN
	4 days	6 days	8 days	10 days	20 days	
<i>Penicillium</i> sp. ....	++	+++	+++	+++	Plate cleared	days 7-8
<i>Penicillium expansum</i> .....	-	++	+++	+++	+++	16
<i>P. pinophilum</i> .....	-	+	++	+++	+++	16
<i>P. atramentosum</i> .....	-	+	++	+++	+++	16
<i>P. chrysogenum</i> .....	++	+++	+++	Fades	++	16-20
<i>P. roqueforti</i> .....	++	+++	Fades	++	++	20
<i>P. citrinum</i> .....	+	++	+++	Fades	++	20
<i>Rhizopus nigricans</i> .....	++	+++	Fades	-	-	
<i>Coniothyrium</i> sp.....	++	++	++	++	-	
<i>Zygorrhynchus moelleri</i> .....	-	++	+++	Fades	-	
<i>Penicillium stoloniferum</i> . . . .	-	++	++	++	Fades	
<i>Mucor</i> sp.....	-	-	+	+	Fades	

+, slight (zones approximately 1.5 to 2.0 cm. in diameter). ++, moderate (zones 3.0 to 5.0 cm. in diameter). +++, abundant (zones 6.0 to 8.5 cm. in diameter).

consisting of 1 gram each of potassium phosphate (dibasic), magnesium sulphate, sodium carbonate, and ammonium sulphate. With the addition of the China blue-aurin indicator the medium becomes of a bright red color. Both the liquid and solid forms of the medium were used successfully in the physiological investigation of cellulose-decomposing microorganisms and in soil fertility problems.

The solid medium proved helpful in the isolation and physio-

logical study of cellulose-destroying fungi. Among the most active forms isolated were members of the genera *Fusarium*, *Penicillium*, *Acrostalagmus*, and *Chaetomium*. Table 1 shows the activities of certain soil fungi upon the China blue-aurin-cellulose agar.

To demonstrate the cellulose-fermenting ability of fungi the following method was found to be useful. Seed fibers of the milkweed, sterilized by hot air in petri plates, were moistened with the basic nutrient solution described above, and inoculated with the fungi. Instead of the liquid, a thin agar prepared with the nutrient solution and indicator, may be poured over the mass of cellulose. The plates are kept at room temperature. The progress of the fermentation may be followed by the microscopic method. A technique suggested by Wood (1924, 1926) was employed in the staining. The fibers were fixed in hydroxylamine hydrochloride (1.8 per cent aqueous solution) for fifteen minutes in watch glasses. They were washed in water and stained for forty-five minutes with benzopurpurin (0.1 per cent aqueous solution). After washing, the fibers were mounted on glass slides.

Cellulose destroyers of the genera *Acrostalagmus*, *Penicillium*, and *Chaetomium* were investigated by this method and the results seem to indicate that for fungi the microscopic method furnishes a valuable criterion of physiological efficiency. The mycelium penetrates the cell wall and develops within the fiber, running parallel, frequently in two strands with many so-called "H" formations. Hyphae may be sent out from the interior of the fiber and these break down the cell wall further, crypts appearing along the walls.

The China blue-aurin-cellulose agar is not entirely satisfactory for demonstrating cellulose decomposition by bacteria. For the study of these organisms in soil, large culture tubes were used containing about 20 cc. of the sterile liquid medium. The first series of tubes received an inoculation of 5 cc. of soil suspension (prepared by shaking 25 grams of soil with 200 cc. of sterile water). After four days' incubation at room temperature the medium became of an intense blue and the cellulose showed definite evidences of decomposition. At the end of the fourth

day 5 cc. transfers were made from these tubes to a second series containing fresh liquid medium. Five such series were prepared, each being allowed to incubate four days before transferring. From the last series direct microscopic examinations invariably revealed, as predominant types, species of bacteria which were later identified with the genus *Cellulomonas*. Occasionally, intimate microbial associations were detected by this method, involving certain other organisms. For example, in one series, colonies of yeast cells appeared, the cells occurring in zoöglöeal masses, and enveloping huge numbers of cellulose-decomposing bacteria. Streak plate cultures were made from this material

TABLE 2

*The influence of yeast upon the activities of Cellulomonas in the China blue-aurin-cellulose medium*

	1 DAY	3 DAYS	5 DAYS	6 DAYS	7 DAYS	8 DAYS
Yeast alone.....	—	—	—	—	—	—
Cellulomonas alone.....	—	—	+	+	++	++
Cellulomonas + yeast.....	—	+	++	+++	+++	+++

—, no change in indicator no visible alteration of cellulose. +, blue color appears at surface of medium; cellulose layer begins to decompose. ++, supernatant medium is bright blue and color extends into cellulose layer, particles of cellulose show definite flocculation into large clumps. +++, entire medium becomes an intense blue; cellulose rapidly dissolves leaving small residue of undecomposed material.

using the China blue-aurin-cellulose agar, and definite blue zones developed in four days. It was further observed that the blue zones did not appear at this stage in the absence of the yeast. The bacillus and the yeast were obtained in pure culture and the association was studied further in large culture tubes of the liquid cellulose medium.

The medium was inoculated with 1 cc. portions from heavily seeded suspensions of *Cellulomonas* and yeast in the liquid medium. There were approximately 12,000,000 cells of the former organism per cubic centimeter and 6,000,000 of the latter. Table 2 reveals the effect of the association.

## CONCLUSIONS

1. For the study of cellulose-decomposing bacteria from soil the China blue-aurin-cellulose solution is very useful. Isolations may be made by the streak plate or by pouring plate methods using the solid medium. Microbial associations can be studied fairly effectively by this method and the present studies are being extended to the determination of other accessory influences in the medium, upon cellulose fermentation.

2. The China blue-aurin-cellulose agar gives excellent results with soil fungi. Using this medium, a method has been developed which furnished a very satisfactory criterion for the cellulose-destroying abilities of these organisms.

## REFERENCES

- SANBORN, J. R. 1926 Jour. Bacteriol., **12**, 1.  
SANBORN, J. R. 1926 Jour. Bacteriol., **12**, 343.  
SANBORN, J. R. 1927a Jour. Bacteriol., **13**, 113.  
SANBORN, J. R. 1927b Jour. Bacteriol., **14**, 395.  
WOOD, F. M. 1924 Ann. Bot., **38**, 273.  
WOOD, F. M. 1926 Ann. Bot., **40**, 547.



# THE INHIBITING EFFECT OF STREPTOCOCCUS LACTIS ON LACTOBACILLUS BULGARICUS

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The fact that certain species of bacteria have an inhibiting effect on the development of other species or varieties is too well known to need discussion. This effect is frequently observed on agar plates on which the development of the colonies of one species may be restricted or completely suppressed by a preponderance of colonies of another variety. Marmorek (1902) has shown that a broth in which a hemolytic streptococcus has grown is unfavorable to the growth of this same organism or to that of any other hemolytic streptococcus. McLeod and Govenlock (1921) have demonstrated that pneumococci inhibit such bacteria as coli, dysentery, and staphylococci, as well as other pneumococci, and that this inhibiting property may be destroyed by heating the cultures to 85°C. Gundel (1927) found that coli not only inhibited the growth of anthrax but under certain conditions completely destroyed it. He also found that this effect could be prevented by shaking the culture with such adsorbing materials as kieselguhr, charcoal, etc.

It is usually assumed that the lactic streptococci and bulgaricus, which differ in their action on milk in degree rather than in kind, grow together satisfactorily and even have a mutually favorable action. However, if a bulgaricus culture is used to increase the acidity of ordinary buttermilk the action is slow and may even fail entirely. If milk is inoculated with equal quantities of lactic<sup>1</sup> and bulgaricus cultures or only slightly predominating

<sup>1</sup> For the sake of brevity *Streptococcus lactis* cultures are referred to as lactic.

quantities of lactic a typical bulgaricus fermentation will ensue. If, however, the lactic inoculation greatly predominates, the bulgaricus culture will be suppressed and the fermentation will be of the streptococcus type. These facts are shown in table 1.

This inhibiting property is apparently common to lactic streptococci although some of the cultures used have been more effective than others in retarding the growth of bulgaricus.

TABLE 1

*The influence of varying inoculation on the relative growth of Streptococcus lactis and Lactobacillus bulgaricus in milk*

INOCULATION LACTIC:BULGARICUS	CELLS ON MICROSCOPIC FIELD LACTIC:BULGARICUS	LACTIC ACID AT 24 HOURS
		<i>per cent</i>
1:1	1:3 32	1 46
2:1	1:0 512	1.23
4:1	1:0 074	0 90
6:1	1:0 051	0 87
8:1	1:0 002	0.91
10:1	1:0.025	0.89

TABLE 2

*The retarded development of bulgaricus in a lactic culture*

AGE OF CULTURE	ST. LACTIS	L. BULGARICUS
<i>days</i>	<i>cells per cc.*</i>	<i>cells per cc.†</i>
2	677,000,000	Under 3,000,000
3	468,000,000	Under 3,000,000
5	4,850,000	Under 3,000,000
6	Under 10,000	43,906,000
8		128,690,000

\* Plate count.

† Microscopic count.

These results seem to be at variance with the usual method of isolating bulgaricus from milk. High acid cultures are obtained by allowing milk to stand in a warm place after curdling occurs. Under these conditions cultures of the bulgaricus type usually develop after the lactic fermentation has taken place.

Table 2 shows the results of an experiment in which sterile milk was inoculated with a lactic culture and after twenty-four

hours' incubation at 30°C. was inoculated with bulgaricus and held at 37°C.

The restraining effect of the streptococci diminished as the cells died or the bulgaricus gradually overcame the unfavorable conditions.

The inhibiting effect of the streptococci is not due to the acidity of the medium. Bulgaricus will develop normally in milk acidified to a reaction as low as pH 5.0 or 4.8.

TABLE 3  
*Inhibiting action in milk cultures held at pH 6.0*

AGE OF CULTURE	LACTIC AND BULGARICUS— BULGARICUS CELLS PER FIELD	BULGARICUS ALONE—BULGARICUS CELLS PER FIELD
<i>hours</i>		
3	0 05	3 90
4	0.15	10 25
5	0.15	19 00
6	0 35	60.25
8	2 55	90 70
9	2.15	169.40
10	2.40	Too many to count

TABLE 4  
*The removal of the inhibiting action from a lactic culture neutralized to pH 6.8*

TREATMENT	CLOUDING	CELL
Heated, inoculated with bulgaricus. ....		Lactic
Filtered, not inoculated. ....		
Filtered, inoculated with bulgaricus. ....	++	Bulgaricus
Filtered and heated, inoculated with bulgaricus. ....	++	Bulgaricus

In table 3 is shown the development of bulgaricus in milk cultures in which the reaction was held at pH 6.0 by frequent neutralization of the acid developed.

The lactic culture was allowed to develop and was then inoculated with bulgaricus. A check flask was inoculated with bulgaricus alone and both were held at 37°C. with frequent neutralization to maintain a reaction of approximately pH 6.0.

The development of the bulgaricus in combination with the lactic was almost nil whereas the multiplication in the check flask



was rapid. The inhibition in this case was entirely independent of the acid.

Heating to the boiling point does not destroy the inhibiting property, but it is at least partially removed by filtering through an earthenware or plaster of Paris filter. The results of an experiment which illustrates this point are shown in table 4.

The medium used was digested casein broth in which *bulgaricus* grew rapidly. After the lactic culture had grown for two days the reaction was corrected to pH 6.8; one fraction was heated fifteen minutes in an Arnold sterilizer and another was filtered through a Chamberland filter. Part of the latter fraction was also heated. *Bulgaricus* failed to grow in the unfiltered heated portion but grew luxuriantly in both the heated and unheated filtered fractions.

Some of these experiments suggest the possibility that the inhibiting property of the streptococci is inherent in the cells or is carried with them. A lactic culture in glucose broth was centrifuged until a mass of cells was obtained. This was washed with sterile water and recentrifuged. The water was decanted off and enough of the cells transferred to yeast extract broth to give a heavy suspension.

After heating a few minutes in the Arnold sterilizer, this broth suspension of killed lactic cells was inoculated with *bulgaricus* and incubated at 37°C. *Bulgaricus* grew readily, which indicates that the dead cells at least do not have an inhibiting effect. In the centrifuging and washing the inhibiting principle must have been removed with the supernatant broth and wash water and therefore it must have been a soluble and possibly a diffusible substance. The correctness of this assumption was demonstrated by the preparation of a collodion sac which was partly filled with yeast extract broth and suspended in a flask of the same medium. The sac was inoculated with the lactic culture and incubated for forty-eight hours. The medium surrounding the sac was then inoculated with *bulgaricus*. A check flask was inoculated at the same time and after twenty-four hours the number of *bulgaricus* cells in each flask was determined by the plate method.

The check flask contained 640,000,000 per cubic centimeter

while the flask exposed to the action of any substance which might diffuse from the lactic culture contained only 82,000,000 per cubic centimeter.

It is very evident from this experiment, which has been repeated a number of times with similar results, that a specific substance was produced in the lactic culture which diffused through the collodion membrane in sufficient quantity to inhibit materially the growth of *bulgaricus*.

It is shown in another paper that this substance also has an inhibiting effect on the lactic culture itself and is probably an important factor in limiting the population of the culture.

#### REFERENCES

- GUNDEL, M. 1927 *Cent. f. Bakt., Abt. I*, **104**, 463-473.  
MARMOREK, A. 1902. *Ann. Inst. Pasteur*, **18**, 169-178.  
MCLEOD, J. W., AND GOVENLOCK, P. 1921. *Lancet*, **200**, 900-903.



## SINGLE CELL TECHNIC

### A PRESENTATION OF THE PIPETTE METHOD AS A ROUTINE LABORATORY PROCEDURE

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Procedures for preparing pure cultures from single bacterial cells which have been published during recent years are diverse and numerous. This mass of advice and description does not, however, seem to have appealed sufficiently to bacteriologists to warrant the introduction of a single cell method as a routine laboratory technic. The indifference which is still widely displayed toward the practice may be due in part to the conviction that some of the systems advocated are uncertain and may result in cultures from more than one organism. Antipathy to other systems arises from the expensive equipment required. In addition to these two objections there is a general feeling that any satisfactory single cell method can be acquired only with great difficulty, and used at the expense of much time even after the operator becomes skilful.

A worker wishing to master a single cell technic without previous experience in it will encounter in the literature on the subject many pitfalls which may well lead to the conclusion that single cell isolations are not only uncertain and expensive but also vexatious and laborious. He will find on the other hand the frequently recurring statement that once gained, the method in question is sure and rapid. It is the opinion of the authors that a negative or unsatisfactory result, when the method is tried, is due to omission of detail in many of the short descriptions and to underem-

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phasis of fundamental points in the more complete articles. It is certain that there are different methods, each sound and speedy, and each giving acceptable results in the hands of those familiar with them, but there has not come to our attention in print a full exposition of any one of these. The usual experience is the development of a new or modified method after time-consuming experiment in each of the laboratories where single cell cultures are required.

The inadequacy of existing information was evident in this laboratory when work on problems involving the question of variation demanded the use of cultures from single cells. Printed descriptions were followed word for word without success. Eventually a modified procedure was developed and used with absolute confidence for securing pure strains of spore-formers, lactobacilli, micrococci, coliform organisms, and other forms of small size requiring the making of examinations under a magnification of 950 diameters.

We venture to place our operations on record in detail with the hope of saving the time of those who may wish to perform single cell operations without making the annoying mistakes which many of the previous descriptions invite. Our instructions have already been followed without difficulty by other workers with successful results, and it has been found that once introduced into a laboratory, many uses are discovered for a single cell method of purifying cultures, frequently with a saving of time over plate, dilution or enrichment methods.

This procedure requires a minimum of special equipment. A viable culture can usually be started in less than half a day. The element of chance is reduced to a minimum. It is therefore felt that the particulars are of general interest, and that laboratories will adopt the single cell procedure for routine as soon as its utility and relative simplicity are generally realized.

It will be noted that the operation as we describe it contains nothing distinctly new. The essential points have been culled from some 30 articles on the subject, however, and certain of them have been refined for working under the oil immersion objective. Steps in the procedure as they appear under the microscope,

and which usually defy verbal description, are illustrated photographically.

#### EQUIPMENT, MATERIALS AND PRELIMINARY TECHNIC

##### *Principle of method*

The most acceptable single cell methods are those which permit the examination under the oil immersion lens of the organisms sought. Two schemes were introduced at about the same time for handling microorganisms on the underside of coverslips. Barber (1904) introduced a mechanically operated micropipette and subsequently enlarged upon his method (1907, 1914). Schouten (1905) devised a micromanipulator with which the organisms were transported by means of a mechanically operated glass-loop of micro dimensions. We arbitrarily decided to adopt the Barber micropipette system. It appealed to us as preferable for the isolation of the smaller organisms and spores, and in addition the making of pipettes is less difficult than the manufacture of the minute glass-loops for which Schouten's arrangement calls. The three separate steps in the micropipette method are the making of a minute hanging drop by means of a mechanically moved micropipette, the examination of this drop for number and type of organisms and the transfer of a chosen drop by means of another pipette into sterile liquid medium.

Apart from the early and now obsolete India ink suspension employed by Burri, nearly all schemes, other than those requiring pipettes, call for the isolation of the organism on solid medium and the making of transfers after a colony has developed from it. One may infer that many of those who still use this means choose it on account of fancied difficulties of the pipette method and that the Barber principle has not been given a fair trial. The pipette isolation is decidedly neater, and in our hands permits the expedition which is claimed as the chief advantage of the agar-block-colony plan.

Both Barber and Schouten controlled their pipettes by means of three lead screws operating in guides in three planes. The modern version of the screw-operated pipette holder is exemplified by the

device of Taylor (1923-25). This equipment although exact and serviceable is costly, and cumbersome for bacterial isolations requiring the constant changing of pipettes. We use the instrument described by Chambers (1922), a compact tool based on the lever principle, which is more flexible for the bacteriologist's purposes.

### *Special equipment*

The micromanipulator is the "right-hand" single pipette holder of Chambers. This clamps on the left side of the microscope stage for operating the pipettes from the side into the open left end of the moisture chamber.

The only additional special equipment necessary is a long focus substage condenser. This is in order to provide sufficient illumination for the examinations, taking into account the fact that the moisture chamber is between the objective and the substage condenser. Our condenser focuses the light at a point approximately 20 mm. above the surface of the stage, that is, on the coverslip where the organisms to be examined are placed.

### *Microscope*

The microscope is furnished with 10 $\times$  ocular, 1.8 mm. (oil immersion), 4 and 16 mm. objectives. The oil immersion lens is used for the actual pipettings and examinations, and the other two objectives are used to watch the coverslip films and to facilitate the centering of the pipettes. Since the three objectives are frequently used in rapid succession, it will be found an advantage to have them accurately parfocalized.

The isolation moisture chamber is carried on an independent mechanical stage.

The microscope assembly is detailed by Kahn (1922), who presents the adaptation of the Chambers micromanipulator to bacterial isolations.

### *Lighting system*

Despite the special substage condenser, the efficiency of an ordinary lighting system is greatly reduced by the gap which must be left between the objective and the condenser. We use a 100-

watt gas-filled bulb placed horizontally about 40 cm. from the microscope with the edge of the filament toward the instrument so that the centers of the filament and mirror are at the same height above the bench. A 500 cc. Florence flask filled with water is used between the bulb and the microscope to converge the light on the mirror and to exclude the heat rays. Some of those who have used this source of illumination prefer to soften the light by adding methylene blue to the water in the Florence flask.

The 100-watt bulb should be shielded in order to protect the eyes of the worker from all light not actually entering the water flask. This precaution facilitates the examinations under the oil-immersion lens by providing contrast between the illumination in the microscope and the light of the laboratory. We further sharpen this contrast by operating in a darkened laboratory and by connecting to a 2-way switch the microscope light and a desk light. The latter is used for preparing the pipettes and for the other steps in the procedure away from the microscope. In this way the organisms in the moisture chamber are exposed to light only when they are being examined, and this is the only illumination in the laboratory when the microscope is in use. As each examination is completed the microscope light is switched off and the desk light switched on simultaneously. In this way it is possible to work for several hours at a time with a minimum of eye strain.

#### *Moisture chamber*

Dimensions of 48 mm. long, 23 mm. wide and 20 mm. high, substantially as given by Kahn (1922), were found convenient for the moisture chamber. The walls, of thin window glass, were mounted on an ordinary microscope slide with Canada balsam. An additional piece of glass was placed flat on the slide in the open end, for stability and to prevent moisture on the floor of the chamber from running onto the stage. By constructing the chamber with its open end flush with the end of the slide the full lateral traverse of the mechanical stage can be utilized. After assembling, the chamber was baked carefully in the oven for several



hours. The sides and end were afterwards lined with black filter paper. Proper moisture conditions can be maintained in this way and the capillary tip of the pipette which refracts the light can be readily seen against the black background. The filter paper should be cut slightly lower on the side facing the operator than on the opposite side and end, so that the tip of the pipette is visible from the side.

The walls of the completed chamber should be square and parallel so that the coverslips fit closely. The top edges of the chamber are smeared thickly with stopcock grease (16 parts vaseline, 8 parts gum rubber and 1 part paraffin melted together) so that after the coverslip is applied, the chamber is completely sealed with the exception of the open end, and so that the triple nose-piece can be swung through the immersion oil without altering the position of the coverslip.

### *Pipettes*

The making of pipettes is illustrated by Chambers (1918). Make them from glass tubing of 4 mm. outside and 2 mm. inside diameters. Draw out one end to 1 mm. diameter for about 4 cm. and seal the extremity. Leave the shank about 10 cm. long, firepolish the open end and plug loosely with cotton. Make a scratch half way along the constriction with a file or glass knife to assist in breaking off the capillary tip to be formed at a later stage. Pack the partially finished pipettes in glass tubes 1.5 by 30 cm., plug these with cotton and sterilize by dry heat. Make a quantity in advance. Either pyrex or soft glass may be used for pipettes. The novice will find that the fine capillaries are more easily made from pyrex, but soft glass will save time after the operator becomes adept.

The capillary tips are made one at a time, as used in the manipulator. Dust and cotton particles collect on the points of finished pipettes during storage and interfere with the making of droplets. The capillary ends are made in a microburner. This is a piece of pyrex tubing 4 mm. in diameter drawn out to a capillary point, bent at a right angle and mounted on a board so that the flame is about 5 cm. above the bench. Illuminating gas is

used for the flame and fed past a screw clamp on a rubber tube to the burner. The flame should be adjusted to burn about 2 mm. high. It is well to practise making the capillary pipettes before any isolations are attempted. The operator is usually advised to make the capillary end in the microflame, and afterwards to bend the capillary portion to a right angle above the flame, so that when the pipette is placed horizontally in the mechanical holder the capillary tip will project vertically underneath and towards the coverslip. We combine the operations of drawing out and bending. This reduces the length of the vertical portion of the pipette, thereby giving more vertical freedom in the moisture chamber. Proceed as follows: By means of forceps sterilized in the flame remove one of the unfinished pipettes from the tubes in which they were sterilized. Grasp the plugged end of the shank horizontally in the left hand. Grip the 1 mm. portion of the pipette in the sterile forceps and heat it in the microflame until the glass softens. When the glass begins to give, jerk the forceps sharply upwards. Clip off the bent capillary portion with the sterile forceps about 5 mm. from the bend. Clamp the pipette in the holder.

Made in this fashion a pipette will have an abrupt bend and after it is in place in the moisture chamber it can be easily centered, approximately, in the low power field. The bend must be square, as otherwise there will be difficulty in ejecting the drops on, and removing them from, the coverslip film. Clipping with forceps almost invariably gives a clean, square opening on the capillary end of the pipette. The bore of the capillaries should be about 5 micra. The first practice pipettes may be measured by means of an ocular micrometer and the heating and pulling timed when subsequent pipettes are made, so that the open capillary end has approximately the recommended size. After the first few satisfactory pipettes have been made, the operator can judge from the "tug" at the time of drawing out whether the pipette is satisfactory. Many of the capillaries taper when the drawing and bending operations are combined. The operator can clip the capillary longer or shorter than 5 mm. where he judges the inside diameter to be about 5 micra. Brief practice

will perfect the knack, although the "feel" of the operation must be modified for hard and soft glasses and for different gas mixtures—as for example, for coal gas in comparison with gasoline gas. Care must be taken to maintain sterile the 1 mm. portion of the pipette and the adjacent end of the shank, which otherwise may contaminate the tubes containing suspensions of organisms or culture media.

It is frequently necessary to use pressure for discharging the pipettes and suction for filling them. This is best done by mouth. A rubber tube connected to the shank of the pipette for this purpose, should be at least one meter long to avoid disturbing the adjustment of the pipette when the operator moves his head. It will be found convenient to connect the rubber tube to the mouth by means of a glass tube bent over the ear. This keeps the tube in readiness when it is not actually being used for sucking or blowing, and also prevents the rubber tube from becoming filled with saliva, which runs back into the mouth through the glass ear lug.

Figure 2, plate 1, shows the appearance of the end of the pipette and the opening in it when the microscope is focused on the end of the pipette in place immediately beneath the film on the coverslip. In figure 3, plate 1, and figure 4, plate 2, there can be seen the shadow of the horizontal 1 mm. portion of the pipette.

The Chambers' pipette holder is usually provided with a sleeve for accommodating pipettes made from small tubing. This is discarded when using the larger and more convenient 4 mm. tubing.

### *Preparation of coverslips*

It is generally admitted that the vital point in the pipette method is the treatment given the coverslip on which the isolations proper are made in the moisture chamber. The necessity for painstaking care with this detail cannot be over-emphasized. Success or failure of the whole procedure depends upon the manner in which the grease film is applied to the coverglass.

To begin with, the coverslips must be freed of dirt, lint and grease which if present would interfere with the examinations.

They must subsequently be treated for the following three reasons. Firstly, the microdrops without treatment of the coverslips will spread beyond the confines of the high power field. Secondly, drops made on a dry cover are shallow and the organisms may suffer on account of the more intimate exposure to the atmosphere of the chamber. Thirdly, a grease film on the cover allows a field of minute drops of condensed water to be maintained around the experimental drop, thereby protecting it from drying and serving as an index of the moisture conditions in the chamber. Hence the grease film applied to the cover must fulfil this threefold object. The film must be of a thickness which furnishes deep, hemispherical drops which remain clear and transparent. There must be sufficient grease to prevent spreading, but not so much that streaks become visible under the oil immersion lens. Many using the method prepare a number of coverslips in advance. We prefer to treat them one at a time as required for the reason that one satisfactory film is adequate for an isolation and preferable to a number of ready-made ones which may later be found unsatisfactory on account of some error in technic.

A grease film which meets the requirements is prepared as follows: Prepare in advance grease-free silk for rubbing the films. Silk is preferable to cotton or linen which may leave fibers on the coverslip. Use a yard remnant of crêpe silk cut into six or eight pieces. Render grease-free by boiling in 10 per cent sodium carbonate solution and rinsing in distilled water. Hang to dry where dust is not likely to blow. Store in a clean tin box.

Use a coverslip 22 by 50 mm. of No. 1 thickness for the oil immersion lens. Boil in acid-dichromate cleaning solution. Wash with distilled water, holding in forceps. Dry with grease-free silk. With a dry finger rub over one side of the coverslip just sufficient Chesebrough's Blue Seal vaseline (antiseptic-free) to give the surface the thinnest possible continuous coating. Rub the vaseline-coated side with the silk, using a circular motion, and keeping several thicknesses of the material between the glass and the fingers. Remove most of the grease in this way. Examine the film by oblique reflected light. It should show a blue continuous haze finely crossmarked by the silk. Grip a corner of the cover-

slip in the forceps and pass it through the blue flame of a bunsen burner, *film side down*. A single flaming of about one second suffices. This sterilizes the coverslip and further distributes the grease. The film side must be down during the flaming. This point, mentioned by Schouten (1905) is essential for success but is not specified by other workers. Adjust the burner flame so that it is not quite hot enough for the glass to crack after the passage.

Saturate the filter paper lining of the moisture chamber with warm water. After the coverslip has cooled, attach it securely to the chamber by means of the stopcock grease on the edges of the walls. Mount the chamber in the mechanical stage of the microscope, turn on the 100-watt light, and focus on the film with the 16 mm. lens. Within a minute or two a fine dew should condense on the film from the moisture in the chamber, which appears microscopically as a field of minute discrete droplets each with a regular margin. If this film does not form within a few minutes, run a drop or two of hot, but not boiling, water on the floor of the chamber using a 1 cc. pipette. The film should then form immediately from the vapor. Examine under the 1.8 mm. lens using immersion oil. The droplets should be clear and should not be broken up by grease streaks. After the first ten minutes the droplets should retain their size, neither running together nor evaporating. If the film has been properly made this appearance can be retained for an hour or longer, with no attention other than occasional introduction of a few drops of warm water on the floor of the chamber as the film begins to dry up near the open end. If it is found difficult to maintain the film on account of low humidity in the laboratory atmosphere, the proper conditions can be secured by making a small pool on the floor of the chamber and encouraging evaporation from it onto the film by the heat of an ordinary microscope lamp placed on the foot of the microscope under the stage. The operator should practise making films until these requirements are met. If the proper appearance is not reached shortly after the film is made, and maintained for an hour or longer, it is useless to proceed with the isolations since drops cannot be applied to and removed

from the coverslip satisfactorily, and there will not be sufficient moisture in the chamber to enable the organisms to survive exposure on the cover.

Plates 1 and 2 illustrate satisfactory films. Any film presenting an appearance intermediate between these two will serve for isolations. The film in plate 1 contained more grease than that in plate 2; the film of plate 2 received a slightly longer flaming. The film of plate 1 was prepared by one of the authors, who works uniformly close to one end of this allowable extreme of greasing and flaming; the other author, whose practice approaches the opposite extreme, prepared the film of plate 2. The operator should be able to tell, after making and examining a few films, what the proper residual amount of grease before flaming should be, and the time of passage through the flame for optimum results. The errors which must be guarded against are illustrated in plate 3. The film of figure 7, plate 3, contained too much grease and the pass through the flame was too rapid. The result is a film so greasy that the streaks persist when the pipette drops are made. Too little grease and prolonged flaming produced the film in figure 8, plate 3. In this case, although the drops are clear, they coalesce and spread beyond the fields of the high power objectives and their shallowness has an unfavorable effect on the organisms. Rubbing the film with a dirty cloth laden with grease from previous work produced the film in figure 9, plate 3. Here pipette drops cannot be examined on account of the debris on the coverslip.

When drops are placed with the micropipette on properly prepared films such as those of plate 1 or plate 2, the outlines of the condensed dew drops originally on the site will disappear and leave a clear drop from the pipette with which the dew drops coalesce. They do not remain outlined in grease after removal. This is brought out in figure 5, plate 2. The field of the oil immersion lens contains about a dozen dew drops with a proper film.

#### *Nutrient media*

A quantity of liquid medium adapted to the organism in question should be dispensed in culture tubes, 18 by 150 mm. This

menstruum, used for the suspension from which isolations are made and also to receive the single cells, should be clear and free from sediment or precipitate. If necessary, the medium should be filtered through a Berkefeld candle.

#### THE ISOLATION

##### *Stock suspension*

Before commencing an isolation there should be on hand supplies of sterile, partially finished pipettes, and clear, sterile liquid medium in tubes, and the operator should be able to make the capillaries and coverslip films with facility. As a stock suspension from which to isolate single cells there should be prepared a young culture of the organism on agar or in broth. In the case of spore-formers an old spore slant may be used. Taking a needle in the case of an agar growth and a pipette in the case of broth growth, transfer sufficient of the material to a tube of the specially cleared broth and shake to distribute the cells uniformly. This should give the medium a turbidity which is just distinctly perceptible.

##### *Pipetting of drops*

Have the moisture chamber in place on the mechanical stage with a good coverslip film. Adjust each of the three fine movements of the micromanipulator to a central position to permit travel in both directions in each of the three planes. Make a capillary pipette. Fill beyond the bend with the suspension of organisms, using suction if necessary. Turn on the microscope light. Place the pipette in the micromanipulator with the vertical capillary end approximately in the optical axis of the microscope. Make the coarse horizontal adjustments by sliding the pipette along in the holder before clamping tight and by turning the pillar in its socket to move the pipette to and fro. Raise the pillar until the tip of the pipette is within 1 mm. of the coverslip. Using only the coarse adjustments bring the tip of the pipette near the center of the field of the 16 mm. objective. For this purpose focus the objective below the cover and look for the shadow of the pipette in the microscope as it is moved gently in the horizontal directions.

When the pipette becomes visible in the low power field, focus on its tip and center and raise it near the coverslip using the fine adjustments of the manipulator and following the movements through the microscope. The three adjustments must be used in turn since raising the pipette may also move it laterally. After centering the pipette in the low power field, center it in the field of the high dry lens. Finally center under the 1.8 mm. lens using immersion oil. The use of the intermediate power may be omitted, although in this case it may be found necessary to move the pipette a short distance with the fine adjustments to bring it in the field of the 1.8 mm. lens.

Retaining the 1.8 mm. objective, focus on the coverslip film. The tip of the pipette should be visible immediately below. Raise the pipette until it just touches the glass, lower immediately and at the same time blow gently. A drop should be left on the coverslip smaller in diameter than the field of the oil immersion objective. The size of the drops can be regulated by varying the length of time of blowing and of the pipette's contact with the cover. The moisture chamber is moved a short distance for each fresh drop by means of the mechanical stage.

The first few drops may contain numerous organisms indicating that the suspension is too heavy. In this case prepare a lighter suspension and repeat the process with a new pipette. If the first few drops do not appear to contain any cells, they may have settled into the lower portion of the capillary. Blow out a large drop to bring this denser suspension into the tip. Continue making smaller drops. When a suspension of the proper density has been used, one or two organisms will be visible in the next few drops made. Continue making microdrops until one is obtained which appears to contain only one cell. The appearance of clear drops with the microscope in focus on the film and the shadow of the horizontal arm of the pipette in the field is brought out in figure 3, plate 1, and figure 4, plate 2.

### *Examination of drops*

All examinations are made under the oil immersion lens. Because the pipette drops are hemispherical rather than flat, the



focusing must be changed continually during an examination so that each drop can be searched thoroughly from top to bottom. A cell may be invisible in the lower part of a drop when the lens is focused sharply on the lower side of the coverglass. Figure 6, plate 2, shows two drops, one free from cells and the other containing a single rod. The microscope is focused below the film on one end of the rod, which is floating obliquely.

### *Removal of organisms*

When a drop has been obtained, definitely containing one cell, turn off the microscope light, lower the pipette, remove from the holder and discard.

Make a new pipette, and fill the tip from one of the tubes of sterile medium. Do not fill beyond the bend. Place in the manipulator, center as before and bring into the field of the 1.8 mm. objective immediately below the drop containing the single selected organism. Make a second confirmatory examination of the drop. Raise the pipette until the tip just enters the drop. The drop should be drawn promptly into the pipette by capillarity. The organism may frequently be seen to be carried into the opening of the pipette. If the drop does not run into the pipette at once, apply gentle suction. When the drop has been removed, focus on the film and examine to see whether the organism was taken by the pipette. The site of the drop should be clear. Occasionally a tiny residual drop is left behind. In either case it is possible to decide immediately whether the organism was removed by the pipette. Move the chamber to the right to minimize the danger of touching any part of it with the pipette. Remove the pipette. Transfer the organism to a tube of sterile nutrient medium. To do this, slant the tube of medium and place the pipette in its mouth, tip down, so that only the sterile 1 mm. portion enters the culture tube. Blow out the contents of the pipette. Wash out several times with the medium. Press the tip of the pipette against the tube so that the end breaks off at the filemark and drops into the medium. Wash into the liquid any fragments of glass, to which the organism may be adhering, by tilting the tube. Discard the pipette and repeat the process using a new pipette filled from the stock suspension.

*Precautions*

The necessity of maintaining the moisture films on the coverslip throughout the operations has already been emphasized. The film should be inspected from time to time and warm water dropped into the chamber whenever signs of drying are evident. A vegetative cell once stranded on the coverslip due to the drying of the drop in which it was contained had best be abandoned.

Attention must be paid also to maintaining sterile the 1 mm. portion of the pipette during handling and care must be taken that drops on the film or the sides of the chamber are not brushed when pipettes are being placed in, and removed from the chamber.

Exposure of the organisms to the microscope light should be reduced to the time during which examinations are being made. When a pipette has been filled with a suspension and no drops have been secured containing one satisfactory organism after several minutes of trial, the pipette should be removed and discarded and a new sample of the suspension taken in a fresh pipette.

Many workers maintain a suspension of organisms on the coverglass together with a pool of the nutrient medium so that dilutions can be made in the chamber on the coverslip for filling pipettes. We prefer to use only test tube suspensions, to be diluted if too dense and reinoculated if too light to contain organisms in the ratio of one to the microdrop. In this way the exposure on the coverslip is reduced to a minimum.

It is interesting to note that the percentage of successful transfers is generally higher on a dark, rainy day than in bright, dry weather. Since conditions on a dull day favor the survival of the cells, it is advisable to duplicate them, in so far as is possible, in the laboratory.

It may be thought advantageous to make a series of single cell drops on the coverslip from a single pipette, marking the positions by means of the scale on the mechanical stage. This also increases the exposure of selected organisms on the coverslip. The percentage of successful growths will be found higher if the directions are followed without modification. In this way the

selected organisms are taken from the bulk of the suspension, and after isolation are inoculated into the new medium with a minimum of delay.

The motility and morphology of the organisms can be studied in the microscope, which is of advantage when isolations are made from a culture of doubtful purity. The lighting is adequate for the observer to distinguish between rods and diplococci, and between rods of different sizes, with the facility of any hanging drop preparation. Spores are plainly visible on account of their high refractivity. In general, any cell which does not show some movement, with the exception of the largest non-motile rods, is stuck to the coverglass and useless for isolation. Spores and the smaller non-motile rods are in continual Brownian movement. The larger rods can be seen to quiver under the Brownian influence. Motile rods display a gyrating and undulating movement in the microdrops precisely as in other hanging drop preparations. They may frequently be observed cruising around the edge of the single cell drop. These factors must be taken into consideration in searching a drop for organisms and in making a rough selection of the more viable cells for transferring singly. An organism which is in rapid movement when first released on the coverslip, but which soon restricts or ceases its activity will not be likely to grow. It may be inferred that the cell was injured mechanically during the operation or has succumbed to the light or to the intimate atmospheric contact of the microdrop.

It should be possible to make ten or more isolations without changing coverslips during a period of two hours. We get from one to five successful growths from ten transfers of single cells from the same suspension.

The portion of the coverslip near the closed end of the chamber is usually the most satisfactory area for the isolations. When the coverslip has been in place for an hour or two, the part at the closed end usually becomes saturated with large dew drops and there is increasing difficulty in maintaining the film at the open end. If it is desired to make further isolations after this has occurred, the coverslip may be turned end for end on the chamber. A new film will then form on the unused portion of the glass.

## DISCUSSION

The chief advantage of the pipette method of isolating single cells with a procedure such as the one we have described is the certainty with which the examinations can be made. By working under the oil immersion objective the operator may be sure that when growth results from a transfer, one and only one organism was its source. Exponents of the various agar-block methods criticize the pipette technic as offering too great an opportunity for contamination. Experience with the method will readily show, however, that this objection is without foundation. During the isolation of more than 100 strains we have not encountered a single contamination. Since this is the universal experience of those using the pipette method, it can be said that possible contamination is not a factor in the procedure. It would seem that many of the agar-block methods offer better chances for the appearance of adventitious organisms in cultures.

While it is not possible to follow the development of the isolated cell into a colony when the pipette technic is used, it must also be remembered that when solid films (nearly all of which contain interfering granules) are examined for organisms under a magnification of 440 times or less, there is always the possibility that nearby dormant cells which have escaped notice may afterwards grow and merge with the selected colony without the knowledge of the operator.

Finally, the micromanipulation requires no more time than do many of the methods based on different principles for which celerity is claimed.

*Alternative methods*

For the sake of completeness we include references to the better known procedures, not requiring the use of pipettes. A "harpoon" system for transferring from colonies of known ancestry has been used extensively by Ørskov (1922a, 1922b, 1924), and it is described in various alternative forms by Hort (1919-20), Levinthal (1927), and Stearn and Stearn (1927). A combination of micromanipulation and agar-film sytem has been devised by

Dickinson (1926a, 1926b). Dickinson places the organisms in the moisture film on a thin layer of agar and moves them about in a capillary bead of liquid formed between the film and a micro-needle. This ingenious device permits examinations to be made under the oil immersion lens.

A departure from the usual solid medium method is the one used by Topley, Barnard and Wilson (1921). This also permits examination under high power. The organisms are placed on a solid medium under quartz covers. Isolated organisms are covered with a bubble of mercury and the unshielded remainder are subjected to ultra-violet raying to destroy all other cells. This method, however, does not appear to effect a saving of time in comparison with micromanipulation, and care is necessary to make sure that all organisms but the one required are killed.

Dark field illumination has been used for making isolations, but in this case the presence of the isolation chamber complicates the problem of lateral lighting. Péterfi and Wámoscher (1926), and Péterfi (1926-27), who have adapted the dark field microscope for micromanipulation, recommend a chamber 4.5 mm. in height for high magnifications. In this restricted space the manipulation of the pipette becomes difficult. A 10 mm. chamber can be used for large organisms, such as yeast, which can be handled under the lower magnifications, as is done by Hahn, Schutz and Wámoscher (1926).

#### *Accessory equipment*

We believe that the pipette method as we have described it can be applied successfully with any type of microorganism. A satisfactory proportion of growths was obtained with the pipette method by Barber (1920) and by Starin (1924) in the case of the anaerobes.

Organisms which cannot be grown in a clear liquid medium can be suspended in an indifferent menstruum for the purpose of isolation, and in this case it is advisable to reduce the actual time required for an isolation to a minimum. The most direct method of attaining this end is by the use of two micromanipulators so

that an isolated organism expelled in a drop by one pipette can be immediately taken off with the other and removed to a suitable culture medium. We believe that the simpler single pipette system will be found inexpensive and convenient for most work, but if the transfers fail to grow when it is used, the apparatus described by Wright and McCoy (1927) is recommended. This is a special form of the double manipulator for bacteriological work. By the use of this machine the length of time during which the organism is exposed on the coverslip can be reduced to the few seconds required for complete examination of the drops.

When warm stage isolation is indicated, as in the case of the gonococcus, the device of Péterfi (1927) may be utilized. Péterfi has perfected a warm stage for micromanipulation of tissue and for the isolation of bacteria. A thermoelectric regulator keeps the temperature of the moisture chamber at 37 degrees.

If organisms are encountered which repeatedly fail to grow after isolation, despite the use of double manipulators and a warm stage, it may be advantageous to circulate air containing approximately 5 per cent carbon dioxide through the moisture chamber. It is advisable to pass the sterilized gas through a bubbler containing warm sterile water before allowing it to enter the chamber. This suggestion is based on the general favorable effect of carbon dioxide on bacteria and bacterial products which has been reported recently by Valley and Rettger (1927) and Valley (1928).

#### SUMMARY

1. The pipette method of procuring single cell bacterial cultures is described in a form which can be applied as a routine procedure requiring a minimum of special equipment.

2. Attention is directed to the steps in the technic which are essential for success and certain refinements are offered.

3. Accessory equipment recently devised by various workers and of service in work with difficultly cultivable organisms is reviewed briefly.

## REFERENCES

- BARBER, M. A. 1904 A new method of isolating micro-organisms. *J. Kans. Med. Soc.*, **4**, 487.
- BARBER, M. A. 1907 On heredity in certain micro-organisms. *Kans. Univ. Sci. Bulletin*, **4**, 3-48.
- BARBER, M. A. 1914 The pipette method in the isolation of single micro-organisms and in the inoculation of substances into living cells. *Philippine Jour. Sci.*, **9B**, 307-58.
- BARBER, M. A. 1920 Use of the single cell method in obtaining pure cultures of anaerobes. *Jour. Exp. Med.*, **32**, 295-312.
- CHAMBERS, R. 1918 The microvivisection method. *Biol. Bull. Marine Biol. Lab.*, **34**, 121-36.
- CHAMBERS, R. 1922 New micromanipulator and methods for the isolation of a single bacterium and the manipulation of living cells. *Jour. Infectious Diseases*, **31**, 334-43.
- DICKINSON, S. 1926-a A simple method of isolating and handling individual fungal spores and bacteria. *Ann. Botany*, **40**, 273-4.
- DICKINSON, S. 1926-b A method of isolating and handling individual spores and bacteria. *Proc. Roy. Soc. Med.*, **19**, 1-4.
- HAHN, M., SCHÜTZ, F., AND WAMOSCHER, L. 1926 Hefe-Ein-Zell-Kulturen mit dem Mikromanipulator. *Z. Hyg. Infektionskrankh.*, **106**, 191-201.
- HORT, E. C. 1919-20 The cultivation of aerobic bacteria from single cells. *Jour. Hyg.*, **18**, 361-8.
- KAHN, M. C. 1922 Chambers' micromanipulator for the isolation of a single bacterium. *Jour. Infectious Diseases*, **31**, 344-8.
- LEVINTHAL, W. 1927 Das Ein-Zell-Kulturverfahren mit der "Objektträgerwanne." *Z. Hyg. Infektionskrankh.*, **107**, 380-6.
- ØRSKOV, J. 1922-a Procédé pour le culture à l'état de pureté d'un élément unique. *Compt. Rend. Soc. Biol.*, **86**, 221-2.
- ØRSKOV, J. 1922-b Method for the isolation of bacteria in pure culture from single cells and procedure for the direct tracing of bacterial growth on a solid medium. *Jour. Bact.*, **7**, 537-49.
- ØRSKOV, J. 1924 Ueber Bakterienreinzüchtung. *Centr. Bakt. Parasitenk.*, I Abt., Orig., **92**, 312-5.
- PÉTERFI, T. 1926 Die Präparier-Wechsel-Kondensoren und ihre Handhabung bei Dunkelfeld-Manipulationen. *Z. Wiss. Mikroskop.*, **43**, 186-214.
- PÉTERFI, T. 1927 Die heizbare feuchte Kammer. *Z. Wiss. Mikroskop.*, **44**, 296-308.
- PÉTERFI, T., AND WAMOSCHER, L. 1926 Die Isolierung von Bakterien im Dunkelfeld: Ein-Zell-Kulturen und Tierimpfung mit einem einzelnen Bacterium. *Z. Hyg. Infektionskrankh.*, **106**, 191-201.
- SCHOUTEN, S. L. 1905 Reinkulturen aus einer unter dem Mikroskop isolierten Zelle. *Z. Wiss. Mikroskop.*, **22**, 10-45.
- STARIN, W. A. 1924 Pure cultures of *Cl. botulinum* from single cells. *Jour. Infectious Diseases*, **34**, 148-58.
- STEARN, E., AND STEARN, A. 1927 Modification of the Ørskov single cell technic. *Jour. Lab. Clin. Med.*, **13**, 276-8.

- TAYLOR, C. V. 1923-25 Improved micromanipulation apparatus. Univ. Calif. Pub. Zool., **26**, 443-54.
- TOPLEY, W. W. C., BARNARD, J. E., AND WILSON, G. S. 1921 A new method of obtaining cultures from single bacterial cells. Jour. Hyg., **20**, 221-226.
- VALLEY, G. 1928 Carbon dioxide studies. III. Preservation of alexin in carbon dioxide: The nature of alexin preservation. Jour. Immunol., **15**, 325-334.
- VALLEY, G., AND RETTGER, L. F. 1927 The influence of carbon dioxide on bacteria. Jour. Bact., **14**, 101-37.
- WRIGHT, W. H., AND MCCOY, E. F. 1927 An accessory to the Chambers apparatus for the isolation of single bacterial cells. Jour. Lab. Clin. Med., **12**, 795-800.



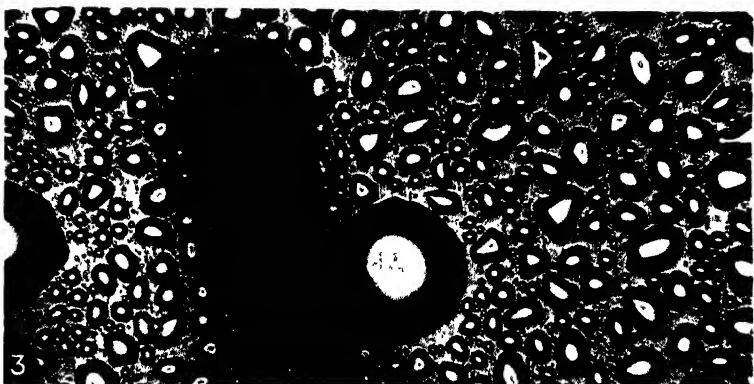
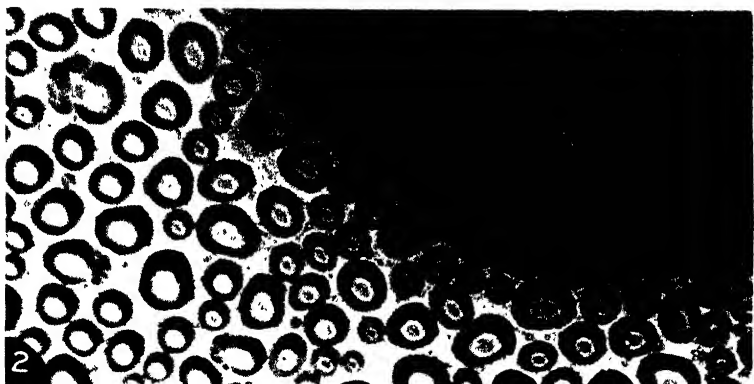
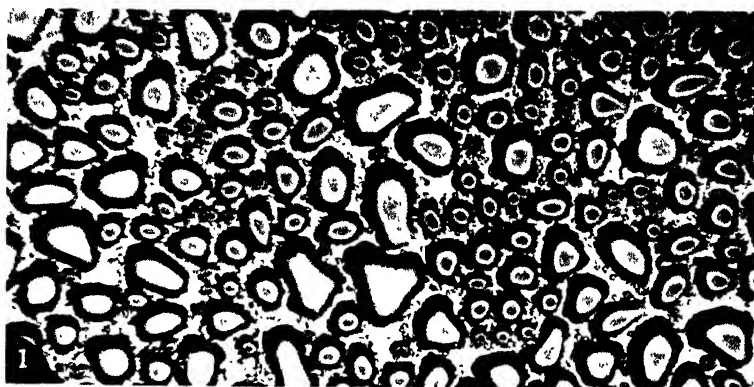
## PLATE 1

## SATISFACTORY FILM, MAXIMUM AMOUNT OF VASELINE

FIG. 1. Film of dewdrops.

FIG. 2. End of pipette just below film.

FIG. 3. Microdrop of sterile broth and shadow of receding pipette.



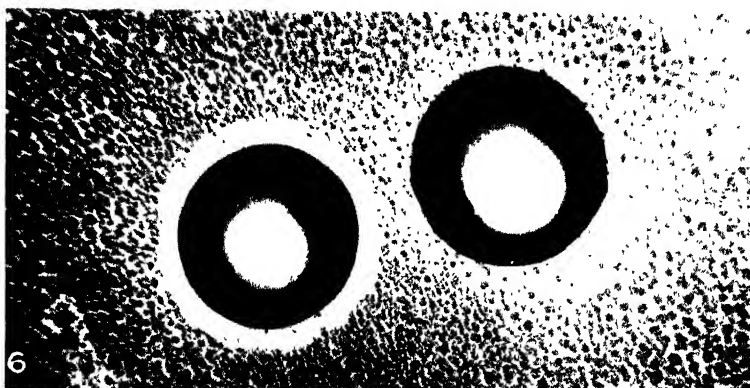
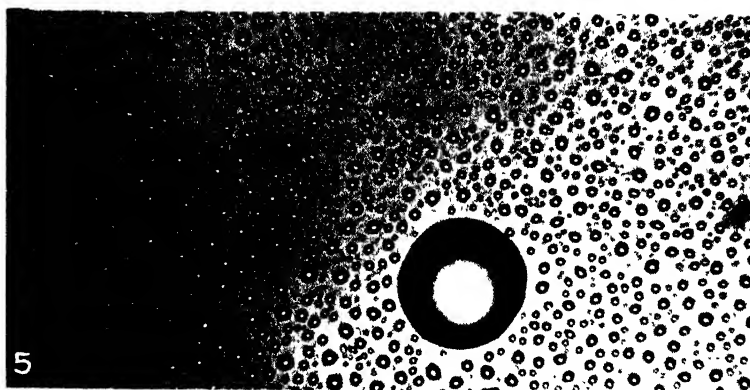
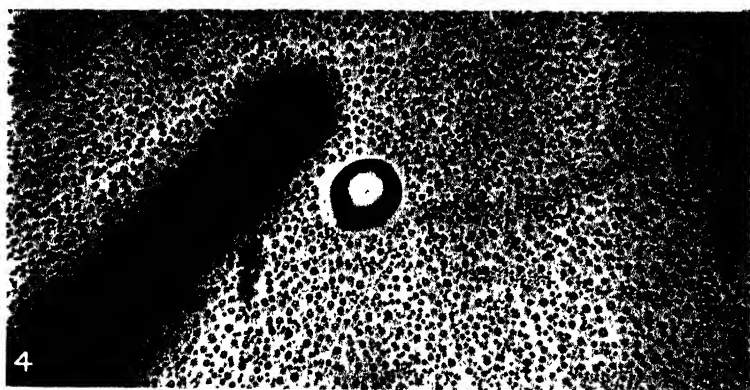
## PLATE 2

## SATISFACTORY FILM, MINIMUM AMOUNT OF VASELINE

FIG. 4. Microdrop of sterile broth and shadow of receding pipette.

FIG. 5. Left, site after removal of microdrop; right, sterile microdrop.

FIG. 6. Left, sterile microdrop; right, microdrop containing a single bacillus



(Gee and Hunt Single Cell Technique)

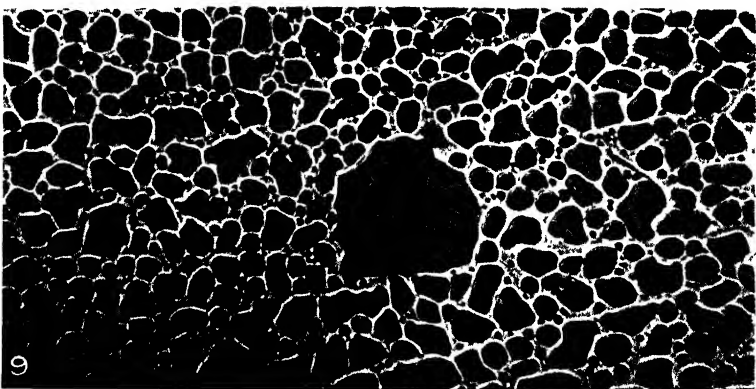
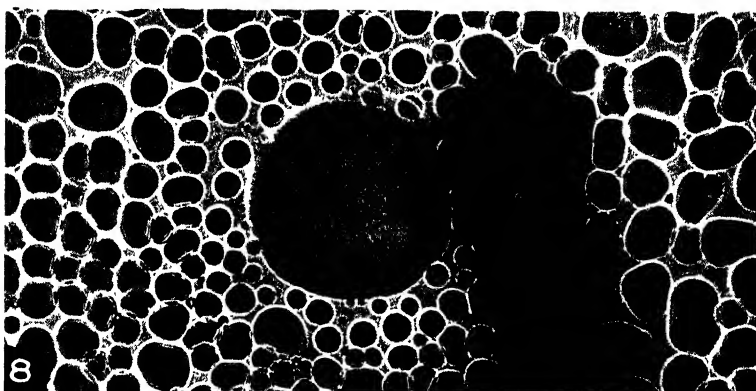
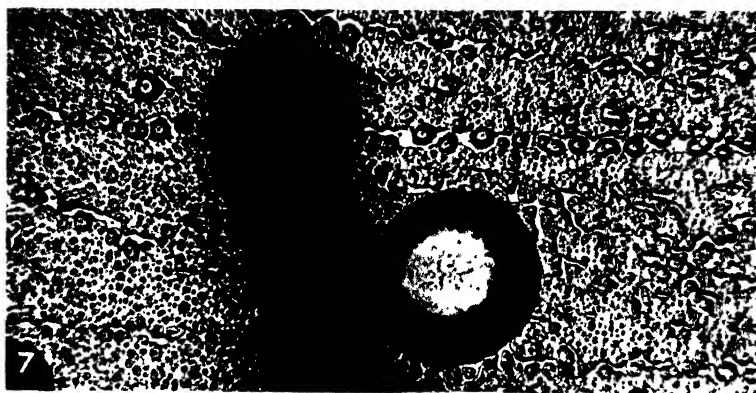
## PLATE 3

## FILMS USELESS FOR ISOLATION PURPOSES

FIG. 7. Excess of vaseline, distortion of dewdrops, streaks in microdrop.

FIG. 8. Insufficient vaseline, spreading of dewdrops, clear but shallow microdrop.

FIG. 9. Film prepared with dirty silk.





# A STUDY OF RENNIN ACTION<sup>1</sup>

## I. RENNIN PRODUCTION BY *BACILLUS PRODIGIOSUS*

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Most of the work reported in the literature regarding bacterial rennin deals with its distribution or methods of preparation. No systematic attempt has been made, it seems, to study any one kind of bacterial rennin from the standpoint of its behavior compared with the behavior of calf rennin. This method has however been applied in the investigation of plant rennins and has yielded information of interest.

Conn (1892) early showed that certain bacteria isolated by him possessed the ability of clotting milk in a rennin-like fashion. This property, moreover, seemed to be distinct from their proteolytic action, indicating that two enzymes were at work. Gorini (1893) found that *B. prodigiosus*, *B. indicus*, *Proteus mirabilis*, and *Ascobacillus citreus* possessed a rennin-like action in milk. He grouped the microorganisms which he investigated on the basis of their ability to ferment lactose and peptonize milk. Wood (1893) reported that *Vibrio cholera* formed a rennin-like enzyme. Kalisher (1900) later added *B. amylobacter* to the list of rennin formers. Loeb (1902) has studied rennin production by *Staphylococcus quadrigiminius* and reported on some of its properties. Finizio (1903) and Savage (1904) advanced some evidence that *Bacillus coli* also produces a rennin-like substance. This observation is of interest because of the low degree of proteolytic activity manifested by *B. coli* and closely allied forms. Hata (1906) reported a method for the separation of rennin from pro-

<sup>1</sup> Abstract of a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



tein-rich media. O'Heir (1906) used alkali as a means of differentiation between acid and rennin curds. Ayers and Johnson (1910) in a detailed investigation of the effects of bacterial growth in milk proposed a classification of bacteria which did not include the rennin forming bacteria as such. Waksman (1919) studied the production of rennin by certain species of *Actinomyces* and classified them on the basis of coagulation and peptonization of milk. This investigator also noted the formation of rennin-like substance in a synthetic medium. His researches indicate that rennin and protease are distinct enzymes. More recently Gorini (1920) has extended his previous work with respect to lactose fermenting and proteolytic bacteria. Waksman (1922) has also called attention to the relative effect of temperature on the interaction between rennin and proteinase with casein. Conn (1922) has reported a new method for the detection of bacterial rennin. Frazier (1925) has studied the production of rennin in milk and has devised a method for the separation and purification of the substance.

In the following pages are recorded some observations on the rennin of *Bacillus prodigiosus*. These experiments fall into the following groups:

1. Rennin production in protein media
2. Rennin production in synthetic media
3. Effect of temperature on rennin formation
4. Relation of cells to clotting
5. Heat inactivation
6. Effect of milk volume on clotting time
7. Influence of rennin volume on clotting time
8. Inhibition by oxalates
9. Coagulation of heated milk
10. Effect of calcium chloride
11. Relation of temperature to the clotting process
12. Effect of cephalin on clotting of milk by rennin

#### 1. RENNIN PRODUCTION IN PROTEIN MEDIA

In an attempt to find a suitable medium for rennin production, peptone, gelatin, and casein solutions have been tried. The protein concentration was varied from 0.1 to 1.0 per cent. It was found that when the organism in question was grown in such media

for a period of from two to three weeks, the cultures, when treated with phenol and mixed with milk in equal parts, induced clot formation. The time required to bring about clotting varied somewhat but on the whole was rather long, from three and one-half to five and one-half hours being necessary in most instances. These results however showed that rennin-like substances were formed from complex proteins alone. The delayed clotting time, it seemed, might be due to the nature of the media or to the presence of inhibitory substances, either in the way of products produced or in the way of excess or unused protein. For this reason it was deemed highly desirable to use a simpler medium of definite composition.

## 2. RENNIN PRODUCTION IN SYNTHETIC MEDIA

Diehl (1919) in his work on proteolytic enzymes used a medium of definite composition with which he obtained growth as well as enzyme formation. The writer has tried various synthetic media, but has found the one used by Diehl to be more satisfactory than most others. This medium has been modified somewhat to suit conditions. As used in these experiments it has the following composition on a liter basis:

$\text{Na}_2\text{HPO}_4$ .. . . . . .	0.50 grams
$\text{K}_2\text{HPO}_4$ .. . . . . .	0.25 grams
$\text{MgSO}_4$ .. . . . . .	0.50 grams
Glycerol .. . . . . .	20 cc.
Nitrogen compound.....	0.5 grams
Distilled water .. . . . . .	1000 cc.

The distilled water was first treated with ammonia-free permutit for twenty-four hours to render it as nearly free from ammonia as possible. The various media were tubed in 20 cc. quantities in large tubes and sterilized in the autoclave. The following nitrogen compounds have been used: Glycocoll, alanine, phenylalanine, histidine, tyrosine, aspartic acid, ammonium tartrate, ammonium lactate, ammonium succinate, ammonium phosphate. In each instance the reaction was adjusted to pH 7.2. The inoculum consisted of a loopful of a twenty-four hour culture of *Bacillus prodigiosus* in the above medium with am-

monium phosphate as the source of nitrogen. Incubation was carried out at 20°C. for fourteen days. The clotting time (C. T.) was determined at room temperature, using 1 cc. of culture and 1 cc. of phenol milk (milk 40 cc. + 2 cc. 10 per cent phenol). The results are set forth in table 1.

Inspection of the table shows that under the conditions of the experiment, ammonium salts, as well as amino acids, serve as sources of nitrogen in maintaining growth of the organism. Also there was formed in these media a substance capable of clotting milk. This substance moreover is destroyed by boiling. A

TABLE 1  
*Clotting of milk by cultures of Bacillus prodigiosus*

SOURCE OF NITROGEN	CLOTTING TIME	HEATED CONTROL	GROWTH
	<i>minutes</i>		
Glycocoll. . . . .	—	—	+
Alanine. . . . .	40	—	+
Phenylalanine. . . . .	—	—	+
Tyrosine. . . . .	48	—	+
Histidine. . . . .	15	—	+
Asparagine. . . . .	80	—	+
Aspartic acid. . . . .	40	—	+
Glutamic acid. . . . .	45	—	+
Ammonium tartrate . . . . .	75	—	+
Ammonium lactate. . . . .	Soft	—	+
Ammonium succinate. . . . .	35	—	+
Ammonium phosphate . . . . .	55	—	+
No nitrogen. . . . .	—	—	—

certain source of error however is apparent in the absorption of ammonia from the air as well as in the carrying over of ammonium ions with the inoculum. In view of the fact that a control containing no nitrogen failed to support growth, it would seem that the error due to small amounts of ammonia in the amino acid media, is of negligible importance. Hence we believe that certain amino acids, as well as ammonium salts, sustain the growth of *Bacillus prodigiosus* and fulfill the requirements for rennin accumulation. The clotting time is a variable quantity and more recent observations have brought out the fact that using the fore-

going medium, with ammonium phosphate as the source of nitrogen, wide variations in clotting time may be encountered. The underlying cause for this has not been found, but when discovered will aid materially in a proper control of conditions favoring rennin production.

### 3. RELATION OF TEMPERATURE

Using the medium described by Diehl and containing 1.75 gram  $(\text{NH}_4)_2\text{HPO}_4$  per liter, adjusted to pH 7.2, temperature effects were studied. Two lots of media were inoculated with *Bacillus prodigiosus* and incubated at 37°C. and 20°C. respectively. The clotting time was determined in the same manner as in the previous experiment. The results are shown in table 2.

TABLE 2  
*Effect of temperature on rennin formation*

AGE OF CULTURE	CLOTTING TIME	
	Incubated at 37°C.	Incubated at 20°C
<i>days</i>	<i>minutes</i>	<i>minutes</i>
3	—	—
6	60	—
8	45	—
14	35	50
Boiled culture	—	—

—, no coagulation.

We note here that an incubation temperature of 37°C. results in a more potent milk clotting culture than does an incubation temperature of 20°C. This is no doubt associated with increased growth since the cultures at the higher temperature grew more rapidly, as evidenced by comparative rates of clouding of the media.

### 4. EFFECT OF CELLS ON CLOTTING

In the foregoing experiments the clotting time of milk was determined using equal parts of phenol milk and whole culture. The use of liquid culture has a disadvantage in that bacteria are added together with bacterial products. This raises the question:

Do fluid cultures of *Bacillus prodigiosus* clot milk by virtue of a milk clotting substance, or is the clotting dependent upon the cell content of the medium? This question has been answered in three ways:

- a. By studying the clotting of milk by centrifuged cultures.
- b. By testing the filtrates of cultures after filtration through a Berkefeld filter.
- c. By filtration through paper.

In the following experiments the medium used was the synthetic medium already mentioned, containing as sources of nitrogen 1.75 grams  $(\text{NH}_4)_2\text{HPO}_4$  and 1 gram of asparagin per liter. The medium, put up in two liter flasks in liter quantities, was adjusted

TABLE 3  
*Effect of centrifugation on clotting time*

	CLOTTING TIME AT 30°C.
	minutes
Whole culture . . . . .	30
Whole culture, boiled . . . . .	No clot
Supernatant fluid . . . . .	30
Supernatant fluid, boiled . . . . .	No clot
Resuspended sediment . . . . .	90

to pH 7.2 and sterilized in the autoclave. As inoculum, a forty-eight hour culture of *Bacillus prodigiosus* in the same medium was used. After seven days of incubation at 37°C., the reaction was found to be pH 6 and 1 cc. of the culture clotted 1 cc. of milk in thirty-five minutes. The culture was then stored in the ice-box, portions being removed as needed for study.

#### 4A. EFFECT OF CENTRIFUGATION

Ten cubic centimeters of media were centrifuged at a high rate of speed for about thirty minutes. This resulted in an abundance of sediment, the supernatant fluid being only slightly turbid. After removing the liquid, distilled water was added to the sediment, bringing the volume back to 10 cc. The mixture was then

shaken and the clotting time was determined for the supernatant fluid and for the resuspended sediment. The findings are recorded in table 3. Clotting time was determined by adding 1 cc. of the test fluid to 1 cc. of phenol milk (milk 40 cc. + 2 cc. 10 per cent phenol). By gently inclining the tube at intervals, the first stage of clot formation could readily be determined. This is represented by the formation of macroscopic particles of casein and serves as a convenient end point.

Table 3 brings out the fact that removal of the major portion of the cells from a culture does not interfere with its clotting properties. Hence clotting seems to be independent of the cell content of the culture.

TABLE 4  
*Effect of Berkefeld filtration*

	CLOTTING TIME AT 30°C.
	<i>minutes</i>
Whole culture.....	30
Whole culture, boiled.....	No clot
Filtrate. ....	82
Filtrate, boiled.....	No clot

#### 4B. EFFECT OF FILTRATION

About 200 cc. of culture were filtered through a Berkefeld filter. The comparative effect of this procedure is shown in table 4.

It has already been shown that removal of the major portion of the cells does not decrease the clotting properties of the culture. Here we note an increase in the time of clotting as a result of filtration. At the same time there occurs a complete removal of the cells. This suggests that during filtration some of the rennin is removed by the filter. To test this hypothesis, some culture was filtered through coarse filter paper just once. This resulted in but slight reduction of the cellular elements since the filtrate was very turbid. The effect of this treatment is shown in table 5.

Summarizing the results of these experiments, we can state that the prolonged clotting time resulting from filtration of cultures is probably due, not to the removal of the cells, but to the

removal of the rennin-like substance by the filters. Furthermore, removal of the cells by centrifugation does not increase the clotting time. Hence the clotting of milk by cultures is independent of the cell content of the fluid.

### 5. HEAT INACTIVATION

Heretofore we have used two criteria to establish the presence of a rennin-like substance in cultures of *Bacillus prodigiosus*. These are: (1) Clotting of milk by cultures and (2) inhibition of clot formation as a result of boiling the culture. In order to insure the validity of the second criterion, experiments have been carried out to determine if the boiling process results in a destruction of the active agent or whether the observed inhibition

TABLE 5  
*Effect of filtration through paper*

	CLOTTING TIME AT 30°C.
	<i>minutes</i>
Whole culture.....	30
Whole culture, boiled.....	No clot
Filtrate.....	45
Filtrate, boiled.....	No clot

is due to loss of some volatile substance. To this end, two active solutions were used, one a solution of calf rennin and the other a culture of *Bacillus prodigiosus*. These were distilled separately and coagulation tests carried out with the distillate, the residue, and the reunited portions of residue and distillate. In no case did clotting occur, even after the reunited fractions were allowed to stand in contact for some time. From this it seems evident that during the boiling process, both solutions were inactivated in a manner which cannot be referred to the loss of volatile substances.

Thermal death point studies were next undertaken for comparative purposes. The solutions used were Difco rennin 1 per cent, and an active culture of *Bacillus prodigiosus* in the synthetic medium previously discussed. Small test tubes, of as nearly

uniform construction as possible, containing 1 cc. quantities of the rennin solutions, were subjected to the desired temperature in a saline bath for 1 minute. The tubes were then removed, chilled, and placed in a water bath at 30°C. and milk was added. In the case of calf rennin, 2 cc. of milk were added and in the case of

TABLE 6  
*Thermal death studies*

TIME OF HEATING	TEMPERATURE	COAGULATION AFTER 1 HOUR	
		Gastric rennin	Bacterial culture
<i>minutes</i>	<i>°C.</i>		
1	40	+	+
1	50	+	+
1	60	+	+
1	70	+	+
1	80	+	+
1	90	+	+
1	100	—	+

TABLE 7  
*Heat destruction at 100°C.*

TIME OF HEATING	COAGULATION AFTER 1 HOUR	
	Gastric rennin	Bacterial culture
<i>minutes</i>		
1	No clot	+
2	No clot	+
3	No clot	+
4	No clot	+
5	No clot	+
6	No clot	+
7	No clot	+
8	No clot	—
9	No clot	—
10	No clot	—

bacterial rennin 1 cc. was used. The reason for this difference will be considered later, but experience indicated that this usage should prevail. Readings were recorded after one hour. Results are shown in table 6. Table 7 brings out the destructive effect of heat at 100°C., the time being a variable.



Inspection of tables 6 and 7 reveals the fact that prodigious rennin is more thermostable than is calf rennin. While an effort was made to maintain the same external conditions at all times, the internal conditions of the fluids were necessarily different. But under these conditions we note that the two types of rennins behave differently.

#### 6. EFFECT OF MILK VOLUME ON CLOTTING TIME

It was noted early that using a fixed quantity of rennin solution and varying the amount of milk, differences in clotting time could be observed. The behavior of the two types of rennin investigated on this basis is set forth in table 8.

TABLE 8  
*Effect of variations in volume of milk*

BACTERIAL CULTURE	MILK	CLOTTING TIME AT 30°C.	CALF RENNIN 1:2500	MILK	CLOTTING TIME AT 30°C.
cc.	cc.	minutes	cc.	cc.	minutes
0.1	0.1	15	0.1	0 1	10½
0 1	0 2	45½	0.1	0 2	7½
0.1	0.3	61	0.1	0 3	4
0.1	0.4	86	0 1	0 4	3½
0.1	0.5	120	0 1	0 5	4
0 1	0 6	151	0.1	0 6	4½
0 1	0.7	180	0.1	0 7	7
			0 1	0 8	10
			0.1	0.9	12

In the case of calf rennin, the ratio of milk to rennin which yields the shortest clotting time is four, while with bacterial rennin this ratio is one. Too much stress should not be laid on a value of four in the case of calf rennin since the value fluctuates somewhat. It has been repeatedly observed, nevertheless, that where calf rennin is added in increasing amounts to a constant volume of milk, the clotting time is shortened up to a certain point, beyond which an increase again occurs. This will be brought out in a subsequent series of experiments. In the light of the present observation it would seem that calf rennin is more susceptible to the concentration of casein than is bacterial rennin. Whether

or not this holds for all concentrations of calf rennin has not yet been determined. This experiment arose from the necessity of determining the relationship involved in answer to the question: How much rennin solution shall be added in each instance? As brought out in the table, the optimum milk-to-rennin ratio is not identical for the two types of rennin. This complicates matters. Where further comparison is needed we have resorted to the use of equal amounts of milk and bacterial culture since this

TABLE 9  
*Effect of rennin variable*

1 CC. MILK + BACTERIAL CULTURE	WATER	FINAL VOLUME	CLOTTING TIME	1 CC. MILK + CALF RENNIN 1:2500	WATER	FINAL VOLUME	CLOTTING TIME
Volume adjusted							
cc.	cc.	cc.	minutes	cc.	cc.	cc.	minutes
0.2	0.8	2	No clot	0.2	0.8	2	No clot
0.4	0.6	2	115	0.4	0.6	2	No clot
0.6	0.4	2	62	0.6	0.4	2	No clot
0.8	0.2	2	42½	0.8	0.2	2	No clot
1.0	0.0	2	31	1.0	0.0	2	No clot
Volume not adjusted							
0.2	None	1.2	246	0.2	None	1.2	12
0.4	None	1.4	81	0.4	None	1.4	9½
0.6	None	1.6	52½	0.6	None	1.6	20
0.8	None	1.8	36½	0.8	None	1.8	107
1.0	None	2.0	31	1.0	None	2.0	No clot

yields the shortest clotting time. For the same reason, we have found it expedient to use one-fourth of this amount of calf rennin.

## 7. EFFECT OF RENNIN VOLUME

Table 9 shows the effects of the rennin volume on the clotting time, the milk volume remaining constant. In one instance the final volume was equalized by the addition of water, in the other, no adjustment was made.

As a general rule, increase in the amount of bacterial rennin brings about a decrease in the time of clotting up to the point

where the volume of bacterial culture equals the amount of milk used. The final limit has not as yet been determined. In the case of calf rennin an increased time of clotting is to be observed where the concentration of rennin is either very large or very small, but intermediate concentrations yield optimum results. This fact has to be considered in attempts to verify the law of Segelcke and Storch. While it is not generally emphasized, the fact remains that this law is operative only under certain restricted conditions. It is hoped to carry out experiments along this line at a later date.

TABLE 10  
*Coagulation of oxalate milk*

VOLUME OF 1 PER CENT $\text{Na}_2\text{C}_2\text{O}_4$ PER 5 CC. OF MILK	VOLUME OF OXALATE MILK USED	VOLUME OF CALF RENNIN USED	CLOTTING AFTER 1 HOUR AT 30°C.	VOLUME OF BACTERIAL CULTURE	CLOTTING AFTER 1 HOUR AT 30°C.
cc.	cc.	cc.		cc.	
0.0	1	0.4	+	1	+
0.1	1	0.4	+	1	+
0.3	1	0.4	—	1	+
0.5	1	0.4	—	1	+
1.0	1	0.4	—	1	+
1.5	1	0.4	—	1	—

#### 8. EFFECT OF OXALATES ON CLOTTING

Soluble oxalates interfere with clotting of milk by calf rennin. This action, while not thoroughly understood, is thought to be due in large part to the removal of calcium ions from solution. Comparative effects of oxalates on the two types of rennin are shown in table 10 and bring out the fact that the bacterial rennin in its function is less susceptible to the restraining action of the salt than is calf rennin.

#### 9. COAGULATION OF HEATED MILK

Milk which has been heated is not as readily coagulated by calf rennin as unheated milk. On the other hand, *Bacillus prodigiosus* readily coagulates milk which has been sterilized in the autoclave. These facts receive support from experiments carried

out on milk heated to the boiling point for one minute. After rapidly cooling to 30°C., rennin was added with the results shown in table 11.

TABLE 11  
*Coagulation of heated milk*

	CLOTTING TIME AT 30°C.
	<i>minutes</i>
Unheated milk + bacterial culture . . . . .	16½
Unheated milk + bacterial culture, heated. . . . .	—
Heated milk + bacterial culture . . . . .	16
Heated milk + bacterial culture, heated . . . . .	—
Unheated milk + calf rennin. . . . .	5
Unheated milk + calf rennin, heated . . . . .	—
Heated milk + calf rennin . . . . .	16
Heated milk + calf rennin, heated . . . . .	—

TABLE 12  
*Effects of calcium chloride on clotting of milk*

VOLUME CaCl <sub>2</sub> PER 5 CC. OF MILK	VOLUME CALCIUM MILK USED	VOLUME OF RENNIN SOLUTION	CLOTTING TIME AT 30°C.
Bacterial rennin			
<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>seconds</i>
0.0	1	1	1800
0.2	1	1	450
0.4	1	1	345
0.6	1	1	390
0.8	1	1	510
1.0	1	1	780
Calf rennin 1:2500			
0.0	1	0.4	52
0.2	1	0.4	33
0.4	1	0.4	40
0.6	1	0.4	55
0.8	1	0.4	77
1.0	1	0.4	130

This brings out another difference between the two types of rennin. The bacterial rennin is capable of clotting heated milk as readily as unheated milk. This is not true of calf rennin. Ac-

cording to Heinemann (1919) heat tends to precipitate the calcium salts of the milk.

#### 10. EFFECT OF CALCIUM IONS

To determine the effect of calcium on the coagulation of milk, the following method was used: To 5 cc. quantities of milk in a series of test tubes were added varying amounts of 0.9 M. calcium chloride, the final volume being kept constant. One cubic centimeter of the calcium milk was then withdrawn and mixed with the rennin solution. The coagulation time was determined at 50°C. The results are shown in table 12.

TABLE 13  
*Effect of heat on clotting of milk*

TEMPERATURE	CLOTTING TIME CALF RENNIN	CLOTTING TIME BACTERIAL RENNIN
°C.	<i>minutes</i>	<i>minutes</i>
10	No clot	No clot
20	35	45
30	5	21
40	3½	10
50	5½	5
60	No clot	No clot

In both instances we note that up to a certain point, calcium chloride hastens clot formation. Beyond this limit it acts as a retarder. Other experiments have shown that the latter effect is only apt to occur where the calcium salt is added in rather concentrated solutions as was done in this instance.

#### 11. EFFECT OF HEAT ON THE CLOTTING PROCESS

It has already been shown that the rennin of *Bacillus prodigiosus* has a higher thermal destruction point than calf rennin. We come next to consider the effect of heat on the clotting time. The method used was to measure out 1 cc. quantities of milk into small test tubes which were then placed in the water bath at the desired temperature. After two minutes, rennin solutions, previously warmed to the same temperature, were added. Findings are reported in table 13.

We note here that as the temperature increases the clotting time decreases. At temperatures higher than 50°C., this relationship soon fails so that at 60°C. no clot formation occurs.

## 12. EFFECT OF CEPHALIN ON CLOTTING OF MILK

Following the early work of Woolridge on the influence of phospholipins on blood clotting, Howell (1912) has shown that the observed effect is due to cephalin. Mills (1921) has brought out the fact that the power of lung extracts to hasten the clotting of blood is due to the same substance. Furthermore he has shown that in these extracts the cephalin occurs in combination with a globulin-like protein. The latter, according to Dorst and Mills (1923), has anti-coagulating properties due to its cephalin binding capacity (Mills, 1926). The antigenic properties of lung and other tissue proteins has been investigated by Downs (1925).

In view of these facts it has seemed pertinent to investigate the effect of cephalin on clotting of milk by rennin. Experiments carried out along this line have revealed the fact that dried gastric mucosa of the calf, when extracted with benzine for a period of nine days, is still capable of clotting milk. The residue obtained after evaporation of the benzine consists to a large extent of fat. If, however, the dried mucosa is first rendered fat-free, the benzine extract upon evaporation yields substances which form a turbid emulsion in saline. This would indicate that removal of the phospholipins does not inactivate the gastric rennin. Other experiments have shown that there occurs no increase in the activity of an aqueous extract of benzine-treated mucosa upon the addition of a saline emulsion of phospholipin from the same source. Emulsions of cephalin derived from brain tissue according to the method of Mathews (1925) as well as commercial cephalin (Difco) fail to increase the speed of clotting of milk by aqueous extracts of benzine-treated mucosa. In some instances it has been found that the clotting time is lengthened following the addition of cephalin emulsion. This may be a physical effect alone or it may be due to removal of calcium by the phospholipin. It is necessary to observe strict precautions with respect to the pH of the solutions in order not to alter the clotting time in this

manner. Experiments dealing with the effect of cephalin on the clotting of milk by cultures of *Bacillus prodigiosus*, point to the fact that here as in the case of gastric rennin, clotting is not enhanced by the phospholipin. The failure of cephalin to shorten the clotting time of milk by both types of rennin points to its unimportance in this connection. This conclusion is supported by the observation that prolonged treatment of gastric mucosa with benzine fails to bring about inactivation.

#### DISCUSSION

It is a noteworthy fact that *Bacillus prodigiosus* is capable of forming rennin in diverse types of media. The formation of rennin in synthetic media containing ammonium phosphate as the sole source of nitrogen is of interest, in the light of the fact that Diehl was able to obtain neither caseinase nor gelatinase in such media. This would seem to indicate that rennin is distinct from proteolytic enzymes. While no attempt has been made to repeat Diehl's experiments systematically, we have observed that filtrates of the ammonium phosphate media have failed to liquefy 5 per cent gelatin. On the other hand, such sterile filtrates do induce some proteolysis of gelatin as shown by Frazier's (1926) plate method. No caseinase has been detected in the filtrates. *Bacillus prodigiosus* brings about little, if any, digestion of casein when grown in milk (Bergey, 1925). While we have thus been unable to differentiate between protease and rennin on a purely qualitative basis, quantitative differences are apparent and indicate that in this instance the two substances are different. The fact that rennin does not follow the rule of specificity implied by Diehl with respect to proteolytic enzymes, points to the relative unimportance of the nitrogen group for its formation. It may therefore be a product of carbon metabolism. The long period of incubation required for rennin production in many instances might lead one to conclude that rennin is a product of cellular autolysis. While this possibility cannot be lightly dismissed, certain facts lend it but little support. Chief among these is our observation that autolyzed cultures do not readily clot milk. Another point in this connection is the fact that rennin yield is variable. Cer-

tain cultures may readily clot milk in four to seven days, whereas other cultures otherwise identical fail to clot milk even after fourteen to twenty-one days. The reason for this difference is not yet apparent and work is being continued in the hope of finding the solution to the problem.

It is difficult to make any generalizations in regard to the comparative mode of action of the two types of rennin investigated. Differences are as a rule quantitative, rather than qualitative. Moreover the observed phenomena vary within certain limits. This variation is determined by the several factors influencing the final result. Thus, for example, rennin concentration, calcium concentration, casein concentration, and temperature are all operative at one time. Attempts have been made by investigators to so standardize conditions that results obtained with calf rennin may be comparable. This effort has not met with much success. The chief difficulty lies in the variation of milk and rennin solutions, not only with respect to concentration of the active ingredients but also with respect to the presence of inhibitory substances. These difficulties become accentuated when comparisons are made between two rennins of diverse sources. We believe however that the use of gastric rennin as a standard of comparison is of value since this substance has been more fully investigated than any other type.

#### CONCLUSIONS

1. Rennin production by *Bacillus prodigiosus* occurs in media containing complex proteins, amino acids, and ammonia as sources of nitrogen.
2. The accumulation of rennin as reflected in the clotting time proceeds more rapidly at 37°C. than at 20°C.
3. Clotting of milk by cultures of *Bacillus prodigiosus* is independent of the cell content of the fluid.
4. Filtrates of cultures are less rennin-active than raw cultures, due perhaps to removal of the rennin by the filter.
5. Prodigiosus rennin is characterized in its action by being more thermostable, less susceptible to oxalates, and apparently less susceptible to the casein concentration than is calf rennin.



6. Both types of rennin are within limits accelerated in their action by calcium chloride and heat.

7 *Prodigiosus* rennin coagulates heated milk more readily than does calf rennin.

#### REFERENCES

- AYERS, S. H., AND JOHNSON, W. T. 1910 The bacteriology of commercial pasteurized and raw market milk. U. S. Dept. Agr. Bureau of Animal Industry Bul. 126.
- AYERS, S. H., AND JOHNSON, W. T. 1913 A Study of Bacteria which survive pasteurization. U. S. Dept. Agr. Bureau Animal Industry, Bul. 161.
- LERGEY, D. H. 1925 Determinative Bacteriology. Baltimore.
- CONN, H. W. 1892 Bacteria in the dairy. 5th Ann. Rep. Storrs Agr. Exp. Sta.
- CONN, H. W. 1892 Isolirung eines Lab-fermente aus Bakterienkulturen. Centralbl. f. Bakt., 12, 223-227.
- CONN, H. J. 1922 A method of detecting rennet production by bacteria. Jour. Bact., 7, 447-448.
- DIEHL, H. S. 1919 The specificity of bacterial proteolytic enzymes and their formation. Jour. Inf. Dis., 24, 347-361.
- DORST, S. E., AND MILLS, C. A. 1923 Comparative studies in blood clotting in mammals, birds and reptiles. Am. Jour. Physiol., 64, 160-166.
- DOWNES, C. M. 1925 The antigenic properties of tissue fibrinogen. Jour. Inf. Dis., 37, 49-52.
- FINIZIO 1903 Kochs Jahresb, 14, 554. Ibid., 14, 532.
- FRAZIER, W. C. 1925 The influence of some bacterial enzymes on heat coagulation of milk. Jour. Dairy Sci., 8, 370.
- FRAZIER, W. C. 1926 A method for the detection of changes in gelatin due to bacteria. Jour. Inf. Dis., 39, 302-309.
- GORINI, C. 1893 Das *Prodigiosus*-Labferment. Hyg. Rundsch., 3, 381.
- GORINI, C. 1902 Ueber die Saure-Labbildenden Bakterien der Milch. Centralbl. f. Bakt. Abt. II, 8, 137-140. Ibid., 55, 240.
- HATA, S. 1904 Über einige Bakterienenzyme und deren Antikörper. Centralbl. f. Bakt. I Ref., 34, 203-209.
- HEINEMANN, P. 1919 Milk. Philadelphia.
- HOWELL, W. H. 1912 The nature and action of the thromboplastic (zymoplastic) substance of the tissues. Am. Jour. Physiol., 31, 1-21.
- KALISHER, O. 1900 Zur Biologie der peptonisierenden milchbakterien. Arch. f. Hyg., 37, 30-53.
- LOEB, A. 1902 Über Versuche mit bakteriellen Lab. und Trypsin. Centralbl. f. Bakt., I, Orig., 32, 471-476.
- MATHEWS, A. P. 1925 Physiological Chemistry. New York.
- MILLS, C. A. 1921 Chemical nature of tissue coagulans. Jour. Biochem., 46, 135-165.
- MILLS, C. A. 1926 The manner of action of the protein fraction of tissue fibrinogen as a blood anticoagulant. Am. Jour. Physiol., 76, 651-659.

- O'HEIR, C. J. 1906 A note on the coagulation of milk by *B. Coli Communis*. Jour. Path. and Bact., 11, 405.
- SAVAGE, W. G. 1904 The coagulation of milk by *B. coli communis*. Jour. Path. and Bact., 10, 90-96.
- WAKSMAN, S. A. 1919 Studies in the metabolism of Actinomycetes. Jour. Bact., 4, 189-216.
- WAKSMAN, S. A. 1922 Enzymes of microorganisms. Abstr. Bact., 6, 265.
- WOOD 1893 Lab. Rept. Royal Coll. Phys. Edinburgh, 12. (Orig. not available.)



# A STUDY OF RENNIN ACTION<sup>1</sup>

## II. EFFECT OF RENNIN ON SODIUM CASEINATE

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Rennin coagulation of milk is not a simple reaction, due in a large measure to the complex nature of the substrate. In an attempt to simplify matters, we have carried out experiments using sodium caseinate. While the results obtained by this method reveal some aspects of the reaction not otherwise apparent, there still remain facts not explicable by prevailing theories. The problem is chiefly one of mechanism of action.

There exists a large volume of literature bearing on this problem. No attempt can be made to review all the literature here but certain workers have particularly emphasized the mechanism involved. In general, attempts to explain rennin coagulation have either a chemical or a physical basis. Thus according to Green (1899), Liebig's explanation rested on the assumption that rennin causes lactic acid to be formed from lactose, the acid precipitating the casein. Hammarsten (1872), (1874), (1877) however showed that no lactic acid was formed, but that two substances, a whey compound and paracasein could be detected as a result of rennin action. Duclaux (1899) failed to observe the whey compound of Hammarsten. Arrhenius (1907) has pointed out that this failure might be due to the adsorption of the compound during its filtration. Bosworth (1913) has shown that the whey compound is in reality a product of autohydrolysis of casein solutions. On the other hand there are some who believe that paracasein is a hydrolytic product of casein. Van Slyke and Bosworth (1913)

<sup>1</sup> Abstract of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

have brought forth evidence to this effect. Inichoff (1922) and Wright (1924), using chemical and physical methods respectively, failed to find evidence of hydrolysis. There are also those who believe that rennin action is essentially a physical change. Fuld (1902) believes the reaction to be a colloidal one. Ringer (1890) has studied the effects of salts on casein and paracasein. His observations were confirmed and expanded by Loevenhart (1904) who concluded that rennin acted in a physical sense rather than a chemical one. Hedin (1909) has suggested that casein and rennin unite by adsorption. This view is shared by Mellanby (1912). Reichel and Spiro (1904) could find but little loss of rennin due to its union with casein. The inactivation of rennin by various means has been investigated by Schmidt-Nielson (1910) and Hedin and Schern (1909). Michaelis and Mendelssohn (1914) have investigated the relation between hydrogen ion concentration and precipitability of casein, before and after treatment with rennin. They point out the analogy between their experiments on casein and acid agglutination of bacteria. Alexander (1912) has investigated the stabilizing effect of gelatin and gum arabic on casein solutions. Palmer and Richardson (1925) believe that casein is a highly stable suspension colloid. They note that casein and paracasein have different acid and alkali combining capacities.

We note here then that there exists no general agreement among the workers in this field with respect to the true nature of the reaction. The difficulty rests to a great extent on the fact that casein is a complex substance and but little understood. For this reason the true explanation will have to await the further development of knowledge in this field. Van Slyke and Bosworth (1913) have pointed out that sodium chloride added to a solution of calcium salt of casein results in a solution of the protein. This they have shown to be a double decomposition and a reversible reaction. They have also shown that as a result of rennin acting upon casein the amount of  $N/50$  HCl required to bring about precipitation is less than in the case of pure casein. They also point out that rennin acts on sodium caseinate without producing a visible change.

In the present investigation a study has been made of the action

of rennin on sodium caseinate. The resulting effect can be brought out by the addition of various salts as well as acids. On this basis we have studied the effects of: 1. Calcium chloride, barium chloride, magnesium chloride, aluminum chloride, and hydrochloric acid on sodium caseinate, before and after treatment with rennin. 2. The effect of salts on sodium caseinate treated with bacterial rennin. 3. The effect of bacterial growth in sodium caseinate as brought out by this method.

#### EXPERIMENTAL

Casein solutions were prepared by dissolving 3 grams of "Casein-Merck- according to Hammarsten" in 8 cc. of  $N/4$  sodium carbonate by grinding in a mortar. About 20 cc. of water were then added and grinding continued until solution was complete. The final volume was then made up to 100 cc. with distilled water. The purest anhydrous salts obtainable were used in these experiments. Stock solutions of the highest concentrations desired were prepared in triple distilled water. These solutions were standardized gravimetrically. Subsequent dilutions were prepared from the stock solution using Ostwald pipettes and burettes and were used without further standardization. The pH of the salt solutions was determined electrometrically using the saturated calomel electrode. These values can have a general significance only and are subject to certain errors common to unbuffered solutions as Clark (1922) has pointed out.

In the experiments on milk, skimmed milk was used to which was added just enough 1 per cent solution of sodium oxalate to restrain coagulation by rennin. The amount of oxalate solution was determined by preliminary titration. The rennin solution used in these experiments was a 1 per cent solution of "Difco rennin 1:30,000."

We have used the following technique in carrying out these experiments. Into each of three beakers, *A*, *B*, and *C* were measured 30 cc. of the 3 per cent sodium caseinate solution already described. To beaker *A* was then added 0.5 cc. of distilled water; to *B*, 0.5 cc. of the rennin solution; and to *C*, 0.5 cc. of boiled rennin solution. After standing at room temperature for

fifteen minutes in order to allow the rennin to act, the contents of each beaker were measured out in 1 cc. quantities into a series of tubes corresponding to *A*, *B*, and *C* above. To each tube was then added 1 cc. of the salt solution of the concentration indicated in the first column of the tables. The final concentration will of course be one-half of this value. The same technical procedure

TABLE 1  
*Showing the effect of calcium chloride*

MOLARITY of CaCl <sub>2</sub>	pH	SODIUM CASEINATE pH 6.7	SODIUM CASEINATE + RENNIN pH 6.7	SODIUM CASEINATE + BOILED RENNIN	MILK pH 6.6	MILK + RENNIN pH 6.7	MILK + BOILED RENNIN
3.76	4.6	3	4	3	—	4	—
1.88	4.8	3	4	3	—	4	—
0.94	4.8	3	4	3	—	4	—
0.094	4.8	4	4	4	—	4	—
0.047	4.8	4	4	4	—	4	—
0.031	4.8	3	4	3	—	4	—
0.023	4.8	1	3	1	—	4	—
0.018	4.8	W	1	W	—	4	—
0.015	4.8	W	W	W	—	4	—
0.013	4.8	O	W	O	—	4	—
0.011	4.9	O	O	O	—	4	—
0.010	4.9	O	O	O	—	4	—
0.0094	4.9	O	O	O	—	4	—
0.0085	4.9	O	O	O	—	—	—
0.0078	4.9	O	O	O	—	—	—
0.0071	4.9	O	O	O	—	—	—
0.0066	4.9	O	O	O	—	—	—
0.0062	5.1	O	O	O	—	—	—
0.0058	5.0	—	O	—	—	—	—
0.0054	4.9	—	O	—	—	—	—
0.0050	4.9	—	O	—	—	—	—

was used in the case of milk. The results will be found in tables 1, 2, 3, 4 and 5. In these and subsequent tables, the degree of precipitation of the protein is indicated by numbers varying from 4 to 1. In salt concentrations less than this the letter *W* signifies that the solution appeared *white* or *milky*. Beyond this, in still lower salt concentrations, the fluid became *opalescent* as indicated by *O*. The degree of opalescence is variable downward, tending

to disappear entirely in the lowest concentrations of salt. This is indicated by (-).

## DISCUSSION

As shown in tables 1 and 2, there is a striking similarity between the action of calcium chloride and of barium chloride. In the first place it is apparent that the addition of the salts to the rennin-free sodium caseinate solution results in precipitation of the protein. In the highest concentrations of calcium chloride this precipitation appears to be incomplete. In concentrations

TABLE 2  
*Showing the effect of barium chloride*

MOLARITY of BaCl <sub>2</sub>	pH	SODIUM CASEINATE + RENNIN pH 6.5	SODIUM CASEINATE + RENNIN pH 6.5	SODIUM CASEINATE + BOILED RENNIN	MILK pH 6.5	MILK + RENNIN pH 6.5	MILK + BOILED RENNIN
0.9400	4.9	4	4	4	—	4	—
0.0940	5.2	4	4	4	—	4	—
0.0470	5.2	4	4	4	—	4	—
0.0313	5.2	3	4	3	—	4	—
0.0235	5.2	1	3	2	—	4	—
0.0188	5.2	W	2	W	—	4	—
0.0156	5.2	W	W	W	—	4	—
0.0134	5.2	O	W	O	—	4	—
0.0117	5.2	O	O	O	—	4	—
0.0104	5.2	O	O	O	—	4	—
0.0094	5.2	O	O	O	—	4	—
0.0085	5.2	O	O	O	—	—	—
0.0078	5.3	—	O	—	—	—	—
0.0071	5.2	—	O	—	—	—	—

of 0.094M to 0.047M calcium chloride precipitation is complete, the precipitated protein undergoing shrinkage, and the supernatant liquid remaining clear. Precipitation decreases with decreasing salt concentration until finally there occurs a zone wherein no precipitate is formed, the fluid appearing white or milky (W). This gives way to an extended zone of opalescence (O) which tends to vanish in the lowest salt concentration. The effect of rennin as shown seems to be to increase precipitation in the higher salt concentrations. That is, the zone of complete precipitation is



broadened and as a result the zones of whiteness and opalescence are shifted in the direction of diminishing salt concentration. It is interesting to note in table 3 that magnesium chloride exerts a solvent effect in high concentrations. This gives rise to another zone, a zone of no precipitate in the highest concentration. In this respect it resembles more nearly aluminium chloride and hydrochloric acid as shown in tables 4 and 5. The two latter substances are distinguished however by the formation of two zones of precipitation. In this instance the effect of the hydrogen ion must

TABLE 3  
*Showing the effect of magnesium chloride*

MOLARITY of $MgCl_2$	pH	SODIUM CASEINATE + RENNIN pH 6.6	SODIUM CASEINATE + RENNIN pH 6.6	SODIUM CASEINATE + BOILED RENNIN	MILK pH 6.6	MILK + RENNIN	MILK + BOILED RENNIN
2.03	—	—	—	—	—	—	—
1.01	5.4	—	—	—	—	—	—
0.101	5.9	2	3	2	—	3	—
0.050	5.9	3	3	3	—	4	—
0.033	5.9	1	3	1	—	4	—
0.025	6.0	W	W	W	—	4	—
0.020	6.0	W	W	W	—	4	—
0.016	5.8	O	O	O	—	4	—
0.014	5.9	O	O	O	—	4	—
0.012	5.8	O	O	O	—	4	—
0.011	5.4	O	O	O	—	4	—
0.010	5.5	—	O	—	—	—	—
0.0091	5.7	—	O	—	—	—	—
0.0084	5.9	—	O	—	—	—	—

be considered and the similarity of action of aluminium chloride and hydrochloric acid suggests that the acidity of the aluminium salt accounts for its peculiar action. While all the salts used are acid, due to hydrolysis, acidity alone cannot account for this behavior. Thus, acids tend to precipitate the protein at its isoelectric point without forming opalescent solutions and if an insufficient amount of acid is added or if the concentration is low the precipitate first formed is redissolved. In the presence of divalent metals such as we have used, a permanent opalescence results. Hence it is an easy matter to detect qualitatively the presence of

rennin in sodium caseinate solutions by adding a dilute solution of calcium chloride and comparing the opalescence with a standard of the same protein concentration. In this way one can readily detect a change of this nature induced by bacteria in sodium caseinate media. In table 7 is shown an application of this method. The organisms were grown in a 3 per cent solution of

TABLE 4  
*Showing the effect of aluminum chloride*

MOLARITY OF $Al_2Cl_6$	pH	SODIUM CASEINATE pH 6.5	SODIUM CASEINATE + RENNIN pH 6.5	SODIUM CASEINATE + BOILED RENNIN	MILK pH 6.5	MILK + RENNIN	MILK + BOILED RENNIN
1 84	1 2	4	4	4	2	4	2
0 92	2 4	—	—	—	—	4	—
0 46	3 1	—	—	—	—	2	—
0 23	3 5	1	1	1	—	2	—
0 115	3 6	1	1	1	1†	2	1
0 092	3 6	1	1	1	1†	2	1
0 018	3 8	2	2	2	1†	2	1
0 0092	3 9	2	3	2	2	3	2
0 0046	3 8	3	3*	3	1	4	1
0 0030	3 9	3	3*	3	—	4	—
0 0023	3 9	3	3*	3	—	1	—
0 0018	3 9	3	3*	3	—	—	—
0 0015	3 9	2	2†	2			
0 0013	4 0	2	2†	2			
0 0011	4 0	2	2†	2			
0 0010	3 9	1	2†	1			
0 00092	4 0	1	2†	1			
0 00076	3 9	—	1	—			
0 00065	4 0	—	1	—			
0 00051	4 0	—	—	—			

\* Curdy precipitate.

† Gelatinous—soft precipitate.

sodium caseinate, pH 6.8, for forty-eight hours. Certain bacteria are able under these conditions to upset the native stability of sodium caseinate with respect to calcium chloride so that the concentration of salt needed for precipitation is reduced. These bacteria are also capable of liquefying gelatin. How shall such results be interpreted? It would seem that proteolytic bacteria

TABLE 5  
*Showing the effect of hydrochloric acid*

NORMALITY OF HCl	pH	SODIUM CASEINATE + RENNIN pH 6.4	SODIUM CASEINATE + RENNIN pH 6.4	SODIUM CASEINATE + BOILED RENNIN	MILK pH 6.5	MILK + RENNIN pH 6.5	MILK + BOILED RENNIN
N	0	4	4	4	4	4	4
1/10	1.01	—	—	—	—	1	—
1/20	1.31	—	—	—	3	2	3
1/30	1.53	—	—	—	1	3	1
1/40	1.60	1	1	1	1	3	1
1/50	1.70	1	3	1	—	3	—
1/60	1.84	2	3	2	—	3	—
1/70	1.84	1	3	1	—	4	—
1/80	1.89	1	2	1	—	4	—
1/90	1.93	—	2	—	—	4	—
1/100	2	—	1	—	—	4	—
1/110	2.08	—	1	—	—	3	—
1/120	2.12	—	—	—	—	3	—
1/130	—	—	—	—	—	2	—
1/140	—	—	—	—	—	—	—

TABLE 6  
*Showing the effect of increasing amounts of rennin*

MOLARITY OF CaCl <sub>2</sub>	SODIUM CASEINATE pH 6.7 + 0.10 cc. RENNIN	SODIUM CASEINATE pH 6.8 + 0.5 cc. RENNIN	SODIUM CASEINATE ALONE pH 6.8
3.76	4	4	3
1.88	4	4	3
0.94	4	4	3
0.094	4	4	3
0.047	3	3	3
0.031	3	3	2
0.023	2	3	W
0.018	1	2	W
0.015	1	1	O
0.013	O	1	O
0.011	O	O	O
0.010	O	O	O
0.009	—	O	—

TABLE 7

Showing the effect of calcium chloride in cultures of bacteria grown in sodium caseinate

ORGANISM	M/40	M/50	M/60
<i>B. coli</i> .....	+	—	—
<i>B. aerogenes</i> .....	+	—	—
<i>B. vulgaris</i> .....	+	+	+
<i>B. cloacae</i> .....	+	—	—
<i>B. pyocyaneus</i> .....	+	+	+
<i>B. vulgatus</i> .....	+	+	+
<i>B. subtilis</i> 133.....	+	+	+
<i>B. subtilis</i> .....	+	+	+
<i>B. prodigiosus</i> .....	+	+	+
<i>M. tetragenus</i> .....	+	+	+
<i>M. aureus</i> .....	+	+	+

TABLE 8

The effect of rennins on sodium caseinate

MOLARITY OF CaCl <sub>2</sub>	SODIUM CASEINATE + WATER	SODIUM CASEINATE + CALF RENNIN	SODIUM CASEINATE + CALF RENNIN BOILED	SODIUM CASEINATE + BACTERIAL CULTURE	SODIUM CASEINATE + BOILED CULTURE
3.76	4	4	4	4	4
0.94	4	4	4	4	4
0.094	3	4	3	4	3
0.047	3	4	3	4	3
0.031	2	3	2	3	3
0.023	1	3	1	3	2
0.018	1	2	W	2	W
0.015	W	2	W	2	W
0.013	W	1	W	W	W
0.011	O	1	O	W	O
0.010	O	1	O	W	O
0.0094	O	W	O	O	O
0.0085	O	W	O	O	O
0.0078	O	O	O	O	O
0.0071	O	O	O	O	—
0.0066	—	O	—	O	—
0.0062	—	O	—	O	—
0.0058	—	O	—	O	—
0.0054	—	O	—	O	—

are capable of altering casein solutions by sensitizing the casein to the precipitating action of the electrolyte. The strain of *B. cloaceae* used in this experiment was an extremely slow gelatin-liquefier. This raises the question as to whether or not the observed action is due to a rennin-like enzyme or to gelatinase. Prolonged growth of bacteria produces such a disintegration of the casein that the sensitization is no longer apparent. This implies that caseinase must also be considered in this connection. In table 8 is shown the effect of addition of a potent milk clotting culture of *B. prodigiosus* to sodium caseinate. The medium used for the preparation of this rennin culture was the synthetic medium described in a previous paper. Such cultures contain but little gelatinase and caseinase. Sensitization occurred here also. If the observed action is due to rennin alone, rennin production seems to be a property common to gelatin liquifying bacteria. This is not always apparent in milk cultures. On the other hand, if gelatinase and caseinase are the enzymes involved, it would seem that previous to proteolysis they combine with casein, thereby producing a change simulating that of rennin. Tables 1 to 5 indicate that the sensitization of casein by rennin occurs in milk as well as in sodium caseinate solutions. The fact that salt concentrations, not effective in precipitating paracasein in combination with sodium, do so where the paracasein exists in combination with calcium (milk), points to the fact that in the former case some of the salt must enter into combination with the paracasein, before precipitation occurs. Since in milk some calcium is already in combination with the paracasein, immediate precipitation occurs at room temperature. But if no rennin is added to the milk the fluid possesses a high degree of stability in the presence of divalent ions. The combination of casein with anions and cations has been investigated by Robertson (1918) and Loeb (1924). These investigators believe that it is unnecessary to invoke the aid of colloidal theories to explain this behavior stating that the observed phenomena obey the ordinary stoichiometric laws. They fail, however, to agree on the point of union of the metallic ion with the protein molecule. But, be that as it may, the fact remains that casein and other proteins are susceptible to the concentration

factor and this holds as we have seen for the reaction of sodium caseinates and paracaseinates with certain salts. From this standpoint these reactions show a striking similarity to the agglutination of bacteria. Buchanan (1918) has brought out the fact that certain types of salts bring about agglutination of bacteria in the absence of immune serum. The addition of specific immune serum reduces the amount of salt needed. In a general way increasing the amount of agglutinin decreases the amount of salt necessary to cause agglutination. These same principles we have observed are operative in the precipitation of sodium salts of casein and paracasein by divalent and other ions.

#### SUMMARY

1. It has been shown that certain salts bring about complete or partial precipitation of casein from solutions of sodium caseinate and that the degree of precipitation is dependent on the salt concentration.

2. Rennin alters sodium caseinate and milk in such a way as to render the protein more readily precipitable by the salts.

3. Certain types of proteolytic bacteria, when grown in sodium caseinate, alter the medium in a similar manner, the resultant change being readily detected by the addition of certain type of salts.

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#### REFERENCES

- ARRHENIUS, S. 1907 *Immunochemie*. Leipzig.
- ALEXANDER, J. 1912 Rennin coagulation of milk from a colloid-chemical standpoint. *Orig. Com. 8th Intern. Cong. Appld. Chem.*, **6** (Sec. 4), 12.
- BOSWORTH, A. W. 1913 The action of rennin on casein. *Jour. Biol. Chem.*, **15**, 231-236.
- BUCHANAN, R. E. 1919 *Agglutination*. *Jour. Bact.*, **4**, 73.
- CLARK, W. M. 1922 *The Determination of Hydrogen Ions*. 2nd Ed. 269. Baltimore.
- DUCLAUX 1899 *Traite de Microbiologie II*. Paris.
- FULD, E. 1902 Über die Milchgerinnung durch Lab. *Beitr. z. Chem. Phys. u. Path.*, **2**, 169.

- GREEN, J. R. 1899 *The Soluble Ferments and Fermentation*. Cambridge.
- HAMMARSTEN, O. 1872 Ueber die Milchgerinnung und die dabei Wirkenden Fermente der Magenschleimhaut. *Maly's Jahresh.*, 2, 118.
- HAMMARSTEN, O. 1874 Ueber den Chemischen Verlauf bei der Gerinnung des Caseins mit Lab. *Maly's Jahresh.*, 4, 135.
- HAMMARSTEN, O. 1877 Zur Kenntniss des Caseins und der Wirkung des Labfermentes. *Maly's Jahresh.* 7, 158.
- HEDIN, S. G. 1909 Über Hemmung der Labwirkung I. *Ztschr. f. Physiol. Chem.*, 60, 85. II. *Ibid.*, 364.
- INICHOFF, G. S. 1922 Über die Chemische Wirkung des Labfermentes. *Biochem. Ztschr.*, 131, 97.
- LOEB, J. 1924 *Proteins and the Theory of Colloidal Behavior*. New York.
- LOEVENHART, A. S. 1904 Über die Gerinnung der Milch. *Zeit. f. Physiol. Chem.*, 41, 177.
- MELLANBY, J. 1912 The coagulation of milk by rennin. *Jour. Physiol.*, 43, 345.
- MICHAELIS, L., AND MENDELSSOHN, A. 1914 Die Wirkungsbedingungen des Labferments. *Biochem. Ztschr.*, 58, 315.
- PALMER, L. S., AND RICHARDSON, G. A. 1925 *Colloid Chemistry of Rennin Coagulation*. *Colloid Symposium Monograph* III, 112.
- RINGER, S. 1890 *Jour. Physiol.*, 11, 464.
- REICHEL, H., AND SPIRO, K. 1904 Fermentwirkung und Fermentverlust. *Beitr. Chem. Physiol. u. Path.*, 6, 68.
- ROBERTSON, T. B. 1918 *Physical Chemistry of Proteins*. New York.
- SCHERN, K. 1909 Über die Hemmung des Labwirkung durch Milch. *Biochem. Ztschr.*, 20, 231.
- SCHMIDT-NIELSON, S. AND S. 1910 Zur Kenntnis der Schüttelinaktivierung des Lab. *Ztschr. f. Physiol. Chem.*, 68, 317.
- VAN SLYKE, L. L., AND BOSWORTH, A. W. 1913 Valency of molecules and molecular weights of casein and paracasein. *Jour. Biol. Chem.*, 14, 227. *Ibid.*, 14, 211.
- WRIGHT, N. C. 1924 Action of rennet and heat on milk. *Biochem. Jour.*, 18, 245.

## A DIAGRAMMATIC SUMMARY OF VARIOUS BACTERIAL CLASSIFICATIONS

A REPORT OF THE COMMITTEE ON TAXONOMY, R. E. BUCHANAN,  
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The publication of a large number of classifications of bacteria in recent years, some by committees of this society, and some by individuals, has led to considerable confusion. Some of this confusion, it is believed, can be cleared up if the various important bacterial classifications are listed in graphic form for comparative purposes. Seven classifications are listed in outline below. To economize in space, definitions and type species are not given here; those desiring such information are referred to the authorities cited.

No preference is implied for any one of these classifications over any of the others or over any classification not here included. It is also plainly to be understood that the Society of American Bacteriologists *has neither adopted nor officially approved* any of these classifications, although two of them have been drawn up by committees appointed by the Society. It must be recognized that this Society has no power to legislate in such matters, and classifications drawn up by its committees must be considered as the work of individuals.

The outlines included here were prepared at the request of this committee by G. I. Wallace and F. W. Tanner of the University of Illinois, except the one on page 393 which is from H. Macy (Chart of the Families and Genera of Bacteria, Jour. Bacteriol., 6, 575-6, 1921).



CLASS SCHIZOMYCETES			
ORDER	FAMILY	SUBFAMILY	GENUS
Eubacteria .....	{	Coccaceae.....	{ Streptococcus Micrococcus Sarcina Planococcus Planosarcina
		Bacteriaceae.....	{ Bacterium Bacillus Pseudomonas
		Spirillaceae .....	{ Spirosoma Microspira Spirillum Spirochaeta
		Chlamydobac- teriaceae .....	{ Chlamydothrix Crenothrix Phragmidiothrix Sphaerotilus
Genera of Doubtful Position .....			{ Spiromonas Spirodiscus Achromatium Newskia
Thiobacteria. ....	{	Beggiatoaceae .....	{ Thiiothrix Beggiatoa
		{ Thiocapsaceae..	{ Thiocystis Thiocapsa Thiosarcina
		Lamprocystaceae	Lamprocystis
		Thiopediaceae...	Thiopedia
		Rhodobacteri- aceae.....	{ Amoebobacter Thiothece Thiodietyon Thioplycoccus
		{ Chromatium Rhabdochromatium Thiospirillum	

Classification from: Migula W. Ueber ein neues System der Bakterien. Arb. a. d. Bakt. Inst. d. Techn. Hochschule zu Karlsruhe, 1, Hft. 2, 235-238. 1894.

## CLASS SCHIZOMYCETES

ORDER	FAMILY	GENUS
Cephalotrichinae. . . . .	Oxydobacteriaceae. . . . .	{ Methanomonas Carboxydomonas Hydrogenomonas Acetomonas Nitrosomonas Nitronomonas Azotomonas
		{ Rhizomonas Corynemonas Mycomonas Actinomyces
		{ Sulfomonas Thiomonas Thiococcus Thiospirillum
	Rhodobacteriaceae. . . . .	{ Rhodomonas Rhabdomonas Rhododietyon Amoebomonas Rhodo'here Rhodopolycoccus Rhodococcus Rhodolacuna Lamprocystis Rhodocystis Rhodocapsa Rhodospirillum
		{ Cladothrix Crenothrix Beggiatoa Thiothrix Leptothrix Spirophyllum Spirochaeta
		{ Denitromonas Liquidomonas Liquidovibrio Liquidococcus Solidococcus
		{ Solidovibrio Spirillum
	Acidobacteriaceae . . . . .	{ Denitrobacterium Bacterium Propionibacterium Caseobacterium Streptococcus Micrococcus Sarcina
		{ Liquidobacterium Bacillus Urobacillus
Peritrichinae . . . . .	Butyribacteriaceae . . . . .	{ Butyribacillus Pectobacillus Cellulobacillus
	Putribacteriaceae . . . . .	{ Putribacillus Botulobacillus

Classification from: Orla-Jensen, S. Die Hauptlinien des natürlichen Bakteriensystems. Centbl. f. Bakt., II Abt., 22, 305-346. 1909.

## CLASS SCHIZOMYCETES

ORDER	FAMILY	SUBFAMILY	TRIBE	GENUS
Eubacteriales..	Coccaceae.....		Streptococ- ceae.....	{ Streptococcus Leuconostoc Diplococcus Neisseria Staphylococcus
			Micrococ- ceae.....	{ Micrococcus Rhodococcus Sarcina
			Siderocap- seae.....	Siderocapsa
			Bacilleae....	Bacillus
	Bacteriaceae .....		Bacterieae..	{ Fusiformis Asterococcus Hemophilus Mycoderma Rhizobium Azotobacter Pseudomonas
				Serratia
				Chromobacterium
				Pasteurella
				Bacterium
				Proteus
Chlamydobacteriales .....	Nitrobac- teriaceae.....			{ Pfeifferella Lactobacillus Erysipelothrix Corynebacterium
				Mycobac- teriae... Mycobacterium
	Spirillaceae .....			{ Vibrio Spirillum Paraspirillum
				{ Nitromonas Nitrobacter Nitrosococcus
				{ Leptothrix Didymehelix Crenothrix Sphaerotilus Clonothrix

Classification from: Buchanan, R. E. Studies on the Nomenclature and Classification of the Bacteria. J. Bact., 2, 155-164, 347-350, 603-617; 3, 27-62, 175-181, 301-306, 403-406, 461-474, 541-545. 1917, 1918.

CLASSIFICATION FROM BUCHANAN—*Continued*

ORDER	FAMILY	SUBFAMILY	TRIBE	GENUS			
Actinomycetales.....				Actinobacillus			
				Leptotrichia			
				Actinomycetes			
				Nocardia			
Thiobacteriales	Achromati- aceae.....			Achromatium			
				Thiophysa			
			Hillhousia				
	Beggiato- aceae.....			Thiothrix			
				Beggiatoa			
				Thioploca			
				Thiocapseeae	Thiocystis		
					Thiosphaera		
					Thiosphaerion		
					Thiocapsa		
					Thiosarcina		
				Lamprocys- teae.....			Lamprocystis
				Thiopédieae			Lampropedia
							Thioderma
				Amoebobac- terieae....			Amoebobacter
							Thiodictyon
							Thiothece
	Thioplycoccus						
				Chromati- eae.....	Chromatium		
Rhabdomonas							
Thiospirillum							
Rhodocapsa							
Rhodothece							
Rhodobac- terioideae.....						Rhodocystis	
						Rhodonostoc	
						Rhodospaera	
						Rhodobacterium	
						Rhodobacillus	
						Rhodovibrio	
						Rhodospirillum	
Myxobacteriales.....				Chondromyces			
				Polyangium			
				Myxococcus			
Spirochaetales.....				Spirochaeta			
				Saprospira			
				Cristispira			
				Treponema			

## CLASS SCHIZOMYCETES

ORDER	FAMILY	TRIBE	GENUS
			Neisseria
			Ascococcus
			Diplococcus
		Streptococceae....	Streptococcus
			Aurococcus
			Albococcus
	Coccaceae.....		
			Rhodococcus
			Micrococcus
		Micrococceae.....	Sarcina
			Nigrococcus
			Nitrobactereae
			Haemophileae
		Graciloideae.....	Graciloides
		Bacilleae.....	Bacillus
		Bacteridiae.....	Bacterium
		Bacteroidae.....	Bacteroides
		Proteae.....	Proteus
			Cloaca
Eubacteriales.....	Bacillaceae.....	Pasteurelleae.....	Pasteurella
			Alcaligenes
			Eberthus
			Shigella
			Dysenteroides
		Ebertheae	Lankoides
			Salmonella
			Balkanella
			Wesenbergus
			Enteroides
			Escherichia
		Encapsulateae.....	Encapsulatus
	Spirillaceae.....		Spirosoma
			Vibrio
			Spirillum
	Chlamydobacteriaceae		
	Mycobacteriaceae.....		Mycobacterium
Thiobacteriales			
Myxobacteriales			

Classification from: Castellani, A , and Chalmers, A. J. Manual of Tropical Medicine. Baillière, Tindall and Cox, London, 1919. See pp. 924-966.

## CLASS SCHIZOMYCETES

ORDER	FAMILY	TRIBE	GENUS
Myxobacterales			
Thiobacterales			
Chlamydobacterales			
Actinomycetales . . . . .	Actinomycetaceae . . . . .		{ Actinobacillus Leptotrichia Actinomyces Erysipelothrix
	Mycobacteriaceae . . . . .		{ Mycobacterium Corynebacterium Fusiformis Pfeifferella
Eubacteriales . . . . .	Nitrobacteriaceae . . . . .	Nitrobacterae . . . . .	{ Hydrogenomonas Methanomonas Carboxydomonas Acetobacter Nitrosomonas Nitrobacter
		Azotobacterae . . . . .	{ Azotobacter Rhizobium
	Pseudomonadaceae . . . . .		Pseudomonas
	Spirillaceae . . . . .		{ Vibrio Spirillum
		Neisseriae . . . . .	Neisseria
	Coccaceae . . . . .	Streptococcae . . . . .	{ Diplococcus Leuconostoc Streptococcus Staphylococcus
		Micrococcae . . . . .	{ Micrococcus Sarcina Rhodococcus
	Bacteriaceae . . . . .	Chromobacterae . . . . .	{ Erythrobacillus Chromobacterium
		Erwiniae . . . . .	Erwinia
		Zopfiae . . . . .	Zopfius
		Bacterae . . . . .	{ Proteus Bacterium
		Lactobacillae . . . . .	Lactobacillus
	Pasteurellae . . . . .		Pasteurella
	Hemophilae . . . . .		Hemophilus
	Bacillaceae . . . . .		{ Bacillus Clostridium

Classification from: C.-E. A. Winslow, Jean Broadhurst, R. E. Buchanan, C. Krumwiede, L. A. Rogers, and G. H. Smith. The Families and Genera of the Bacteria. Final report of the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types. Jour. Bact., 5, 191-229. 1920.

## CLASS SCHIZOMYCETES

ORDER	FAMILY	SUBFAMILY	TRIBE	GENUS
Eubacteriales	Nitrobacteriaceae.....		Nitrobacterieae....	{ Hydrogenomonas Methanomonas Carboxydomonas Nitrosomonas Nitrobacter Nitrosococcus Acetobacter Thiobacillus
				{ Azotobacter Rhizobium
				{ Streptococceae.... { Diplococcus Streptococcus Leuconostoc
				{ Neisserieae..... { Neisseria Gaffky
				{ Micrococceae..... { Staphylococcus Micrococcus Sarcina Rhodococcus
				{ Spirillaceae..... { Vibrio Spirillum
				{ Chromobacterieae { Serratia Flavobacterium Chromobacterium Pseudomonas
				Cellulomonadeae . Cellulomonas
				Achromobacterieae Achromobacter
				Lactobacilleae .... Lactobacillus
	Bacteriaceae ....		Erwinieae .....	{ Erwinia Phytomonas
				Kurthieae ..... Kurthia
				{ Escherichia Aerobacter Proteus Salmonella Eberthella Alcaligenes
				Pasteurelleae .... Pasteurella
				Klebsielleae..... Klebsiella
				Hemophilae..... { Hemophilus Dialister
				Bacteroidese..... Bacteroides
				{ Bacillus Clostridium

Classification from: Bergey's Manual of Determinative Bacteriology. D. H. Bergey, assisted by a committee on the Society of American Bacteriologists,—Harrison, F. C., Breed, R. S., Hammer, B. W. and Huntoon, F. M. Williams and Wilkins, Baltimore. 2nd ed., 1925.

CLASSIFICATION FROM BERGEY—*Continued*

ORDER	FAMILY	SUBFAMILY	TRIBE	GENUS
Actinomycetales....	Actinomycetaceae.....			{ Actinobacillus Leptothrix Actinomyces Erysipelothrix
	Mycobacteriaceae .....			{ Mycobacterium Corynebacterium Fusiformis Pfeifferella
Chlamydothales	Chlamydothaceae.....			{ Leptothrix Didymobolus Crenothrix Sphaerotilus Clonothrix
Thiobacteriales.....	Rhodobacteriaceae .....	Chromatiales .....	Thiocapsae ..	{ Thiocystis Thiosphaera Thiosphaerion Thiocapsa Thiosarcina
			Lamprocystae ..	Lamprocystis
			Thiopediaceae ....	{ Thiopedia Thiodierina Lampropedia
			Amoebobacteraceae	{ Amoebobacter Thiodictyon Thiotheca Thiopolycoccus
			Chromatiaceae ..	{ Chromatium Rhabdomonas Thiospirillum Rhodocapsa Rhodotheca
			Rhodobacteroidae .....	{ Rhodocystis Rhodonostoc Rhodospirillum Rhodobacterium Rhodobacillus Rhodovibrio Rhodospirillum
			Beggiatoaceae.....	{ Thiobacillus Beggiatoa Thioploca
			Achromatiaceae .....	{ Achromatium Thiophysa Thiospira Hillhousea
				{ Myxococcus Polyangium Chondromyces
				{ Spirochaeta Saprospira Cristispira Borrelia Treponema Leptospira



## CLASS SCHIZOMYCETES

ORDER	FAMILY	GENUS	SUB-GENUS
Schizomycetales	Coccaceae.....	Streptococcus	
		Sarcina	
		Micrococcus.....	{ Diplococcus (Gram-positive group)
			{ Nitrosomonas
			{ Nitrobacter
			{ Rhizobium
			{ Haemophilus
			{ Brucella
			{ Pasteurella
			{ (Glanders and dysentery group)
			{ (Photogenic group)
			{ (Aerogenes group)
Schizomycetales	Bacteriaceae.....	Bacterium.....	{ Encapsulatus
			{ (Typhoid group*)
			{ Salmonella
			{ (Coli group*)
			{ Acetobacterium
			{ (Cloacae group)
			{ (Red chromogens)
			{ (Blue and violet chromogens)
			{ Pseudomonas
			{ Proteus
			{ Erysipelothrix†
		Fusobacterium	
		Plocamobacterium	
Schizomycetales	Desmobacteriaceae.	Beggiatoa	
		Leptothrix.....	{ Leptothrix
			{ Chlamydothrix
		Crenothrix	
		Cladothrix	
		Thiothrix	
	Spirillaceae.....	Vibrio	
		Spirillum	
	Spirochaetaceae....	Spirochaeta	
	Bacillaceae.....	Bacillus.....	{ Aerobic group
			{ Anaerobic group
Actinomycetales	Proactinomycetaceae.....	Corynebacterium	
		Mycobacterium	
	Actinomycetaceae..	Actinomyces	

\* In a footnote under these groups the authors refer to the names given by Castellani and Chalmers.

† This subgenus is listed as an appendix to the genus Bacterium.

Classification from: Lehmann, K. B., and Neumann, R. O; assisted by Dold, H., Sussmann, O. and Haag, F. E. Bakteriologische Diagnostik. II Band. J. F. Lehmann, Munich; 7th ed., 1927. See pp. 192 ff.

# AN INVESTIGATION OF STREPTOCOCCI ISOLATED FROM THE ALIMENTARY TRACT OF MAN AND CERTAIN ANIMALS

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The streptococcal organisms to be found in the alimentary canal have been reported upon by several writers. Gordon, in evolving a classification of streptococci by means of the fermentative action of the different strains on seven chemical substances and their ability or inability to clot milk and reduce neutral red, distinguished 48 varieties among 300 streptococci from normal saliva. A. C. Houston, using the same tests, found 40 types in 300 streptococci from normal human feces. Andrewes and Horder recognized that although the chemical types of all the streptococci were connected by innumerable transition forms, yet on the basis of the numerical frequency of certain sets of reactions 6 main types (excluding pneumococci) could be recognized, and the remaining strains described as variants from these by one or more characteristics. The streptococci from the alimentary tract, in their review, were found among the 5 types named equinus, mitis, salivarius, anginosus, fecalis or the variant strains related to them. Holman based a more logical form of classification on the presence or absence of hemolysis and the ability or inability to ferment lactose, mannitol and salicin. The streptococci derived from feces were found almost entirely in 5 of the 16 types derived by this method, viz., *Streptococcus fecalis*, equinus, mitis, pyogenes (names adopted from Andrewes and Horder), and a type named by Holman *Streptococcus infrequens*. Almost half of them were in the *Streptococcus fecalis* group.

The name enterococcus was used by Thiercelin to designate an

organism which he found present in normal stools, and in purulent discharges. In his descriptions it is presented as an organism of very varying morphology, sensitive to heat, not capable of fermenting sugars, and toxic to mice. Later descriptions of a type of organism given the same name do not conform to this, and the name is now bestowed on a large oval or rhomboid coccus appearing in pairs or short chains, exceptionally resistant to heat, capable of fermenting several "sugars" and relatively avirulent to laboratory animals. This type does not exactly coincide with any of those of Andrewes and Horder or Holman, but in reference to their classification it could be described as a well defined subgroup of *Streptococcus fecalis*. The enterococcus has been the subject of extensive investigation by Dible and by Bagger. T. Houston and McCloy described the organism in its relation to febrile illnesses in soldiers during the last war.

Further references to the literature will be made when the results of the present investigations are discussed.

#### SOURCE AND PRIMARY CULTURE

For the work from which the strains used in this investigation were derived, a measured amount of the feces or other specimen, emulsified and diluted if necessary, was cultured in several kinds of medium, aerobically and anaerobically. Streptococci were found most readily on the aerobic plates of 10 per cent rabbit-blood agar and of brom-cresol-purple lactose agar, and most of the strains used in the present work were obtained from these media. Some, also, were obtained from tubes of litmus milk and of Robertson's bullock heart medium, both by anaerobic culture. From whatever primary culture, a routine method was employed in the isolation and examination of the strains.

#### METHODS

Isolation of the streptococci in pure culture was obtained by plating out a single colony of the primary culture on 10 per cent rabbit-blood agar. The presence or absence of hemolysis was noted. A single, well separated colony was subcultured in 0.9

per cent potassium-dihydrogen-phosphate bouillon, pH 7.4. The morphology of the organism in this medium was observed by the use of Kopeloff and Beerman's modification of Gram's staining method.

From the broth culture there were inoculated a plate of ordinary agar and one of McConkey's bile-salt neutral-red lactose agar, to discover whether these media were suitable or not for growth of the organism. To test the power of methemoglobin production, there was used a sloped tube of coagulated sheep's blood, a medium which has been found the most suitable for gauging this property. The power to ferment lactose, mannitol, salicin, glucose, sucrose and inulin was examined by means of slants of serum agar to which were added 1 per cent of the fermentable substance and litmus as an indicator. Final readings of the fermentation tests were made after incubation at 37°C. for twenty-one days.

The question of bile solubility was decided by the addition of 0.1 cc. of a sterile 10 per cent solution of sodium taurocholate to 1 cc. of the broth culture twenty-four to forty-eight hours old, and examination was made after an hour in an incubator at 37°C. In cases of doubt, when the broth culture was not very turbid, a stained film preparation was used to make sure whether or not lysis of the organisms had occurred. Control tests of the solubility of pneumococci were carried out.

To test the heat resistance of the organisms, 2 cc. of a young phosphate broth culture were pipetted into each of two sterile and stoppered Wassermann tubes. One tube was placed in a water-bath at exactly 60°C. for ten minutes, and the other for fifteen minutes. After this immersion the tubes were cooled in water at room temperature, and two large loopfuls of each tube were plated on blood agar.

#### SOURCE OF THE STRAINS

Out of the total of 51 strains, 43 were of human origin, being derived from 17 different persons, in many cases specimens being obtained at different times and from different parts of the alimentary tract from one individual. Thirty-seven of these strains were from the stool, 2 from the duodenum and 4 from the mouth.

Six specimens were obtained from rats (4 from feces and 1 each from ileum and cecum).

Two strains were obtained from the stomach of a dog.

#### STAINING AND MORPHOLOGY

In regard to staining, all the strains were strongly Gram-positive, although in each film a certain number of individual elements were decolorized.

Morphologically, there were found 3 distinguishable types of organisms. One type was composed of strains of almost spherical cocci arranged in medium-length or long chains, and all these strains failed to grow on McConkey's medium, and were not heat resistant in the test described above. They included all the strains which were flocculent or granular on first culture in phosphate broth; but these granular strains usually became less granular on subsequent broth culture. There were 6 strains of this type and all came from the mouth, stomach or duodenum. They will be further described at a later stage.

The second type of morphology was of strains of cocci which appeared in pairs or short chains of 4 to 6 individuals and which showed a distinctly oval shape. With one exception, all these strains grew well on McConkey's medium.

The strains of type III showed in both cultures a more markedly lanceolate shape than those of type II, it was less frequent among the former for more than two cocci to be found attached and often an unstained zone (which could not be stained by Hiss's method) was seen around them and not around the cocci of type II. All but one of type III grew on McConkey's medium.

On media other than broth, such as coagulated sheep's blood medium, the distinction between types II and III could not be so well made out, for some strains which showed mostly lanceolate forms in broth showed many oval forms on solid media. It was found, also, that each of the two types, as judged by examination of broth cultures, contained heat resistant and heat sensitive strains, although type II included only 5 out of 29 (15 per cent) resistant strains, and type III 10 out of 16 (63 per cent). It must be emphasized that the contrasting descriptions given of

these types are pictures of the extreme differences, and that in some cases it was found that a strain does not clearly resemble its type in all particulars. In some cultures short bacillary forms were seen, and they persisted after further plating to ensure purity of the culture. This feature is commented on in regard to streptococci, especially non-hemolytic types, by Holman in connection with Gordon's previous observation of it in *Streptococcus scarlatinae*.

#### GROWTH ON MEDIA

Every strain grew well on blood agar, and none produced hemolysis except no. 7 (a heat resistant strain which grew on McConkey's medium and was isolated from human feces). Among the strains (heat resistant or heat sensitive) which grew on McConkey's medium, it is recorded of many that the largest isolated colonies on blood agar after a few days' growth reached a size of 1.5 to 2.5 mm. and were greyish in color.

Ordinary agar was a suitable medium for every strain, and the colonies varied in appearance from minute colorless specks to greyish round discs, 1 to 2 mm. in diameter.

All the organisms grew rapidly and abundantly in phosphate broth, and the growth produced uniform turbidity, except in the case of 5 long-chained cocci which did not grow on McConkey's medium. These granular strains became less granular on sub-culture.

McConkey's medium was suitable for the growth of all the diplococcal forms (types II and III), except 2 strains. None of the strains showing well marked chain formation grew on this medium. Four of the strains not growing on bile-salt lactose agar were derived from the teeth and 1 from the duodenum of human beings, 2 strains from the stomach of the dog, and 1 from a rat's feces. All of those which grew on McConkey's medium came from feces or directly from the small or large intestine.

Coagulated sheep's blood medium produced a good growth of every strain and methemoglobin was produced by some members of every group, whether subdivision is made by morphology, ability to grow on McConkey's medium or the effect of heat on

viability. In many cases it was slight, but sometimes very well marked and in such cases the bile solubility and ability to grow on McConkey's medium were noted with special care. Dible states that none of the 89 fecal streptococci which he classes as enterococci produced methemoglobin, but my findings differ from his in this respect.

On brom-cresol purple lactose agar plates, from which many of the strains were isolated, the streptococci grew well in colonies of up to 2.5 mm. in diameter, showing by transmitted light a thick central core and a less opaque peripheral zone with a sharp margin. This appearance was found with enterococci and also with non-heat resistant forms.

#### BILE SOLUBILITY

By the method described above, all the organisms were found to be insoluble by bile salts. In the case of thin emulsions the result was tested by staining and examining microscopically for unlysed cocci of normal appearance in large numbers.

#### HEAT RESISTANCE

After ability to grow on McConkey's medium, resistance to heat (60°C. for ten to fifteen minutes) has been the second basis of subdivision of the organisms examined. First, all those which did not grow on bile-salt lactose agar were killed in the heat test, and, of the remainder, 16 strains were heat resistant and 27 not. In 2 cases (nos. 15 and 16) heat resistance was found to be of a lower degree than in the others. This is in line with Dible's statement that like most other tests for the differentiation of streptococci, the heat resistance test is not an absolute one for the recognition of enterococci, although in his experience it is more constant than any other yet introduced.

#### FERMENTATION TESTS

*Glucose* and *sucrose* were fermented by every strain, and *lactose* by all except a long-chained one, from a dog's stomach, which did not grow on McConkey's Medium and was not heat resistant.

*Salicin* was fermented by all except 2 strains, 1 from the teeth and 1 from the duodenum of the same man, and these likewise were long-chained, did not grow on McConkey's medium and did not survive the heat test.

As regards the fermentation of *Mannitol*, 3 out of 8 strains which did not grow on McConkey's medium and were not heat resistant acted on this alcohol, and 5 did not. Of the heat resistant strains, 11 were positive and 5 negative as regards mannitol fermentation. In the group which did not survive the heat test, but grew on McConkey's medium, there were no mannitol fermenters.

*Inulin* was fermented by\* a larger proportion of the strains than was expected. It was split by 1 out of 8 of the strains which

TABLE 1  
*Mannitol fermentation*

	FERMENTATION	NO FERMENTATION
No growth on bile-salt medium .....	3	5
Growth on bile-salt medium heat-resistant . . . . .	11	5
Growth on bile-salt medium <i>not</i> heat-resistant.. . . .	0	27
Total .....	14	37

did not grow on McConkey's medium and by none of the heat resistant organisms, but the majority (21 out of 27) of those which grew on McConkey's medium and did not survive the heat test fermented it. All of these strains were certainly bile insoluble, and in the case of 3 of them their action was tested on two separate specimens of inulin supplied by different chemists, and the results were in agreement.

#### DISCUSSION

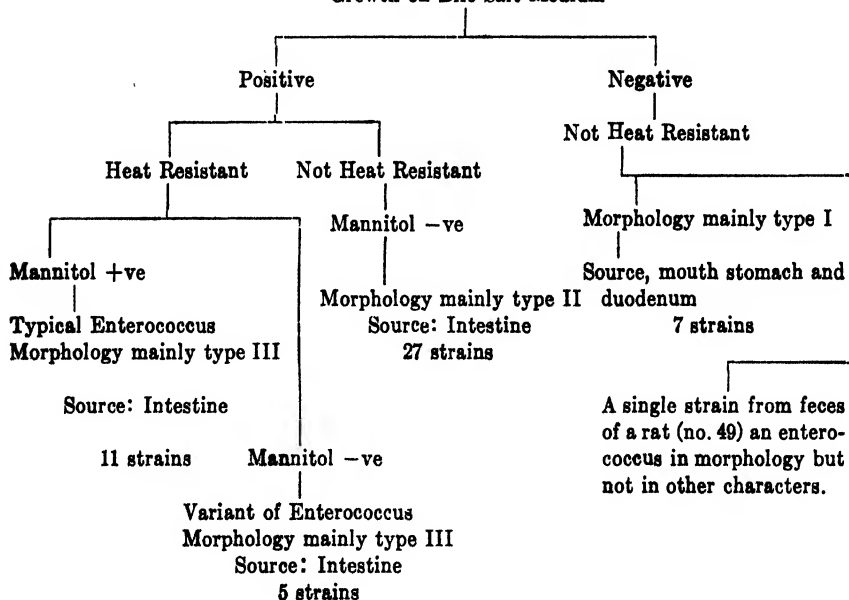
On the basis of three tests, namely, ability to grow on McConkey's medium, heat resistance and mannitol fermentation, the following subdivision of strains may be made, and in the main the subdivisions are associated with the morphological types indicated above and with the source of the organisms (table 2).



In the main, the results of the examination of this series agree closely with the results, recorded and analyzed by Dible, of 152 strains of streptococci from the human bowel.

Andrewes and Horder, in their original description of *Streptococcus fecalis* give it the characters, among others, of being short-chained, with a tendency to long chains in disease, of being non-hemolytic, of fermenting sucrose lactose, salicin, coniferin, mannitol and sometimes inulin and raffinose. They state that

TABLE 2  
Growth on Bile-Salt Medium



in their strains mannitol fermentation was constant, but admit that this power is probably not an absolute criterion, since Gordon found strains which resembles *Streptococcus fecalis* in all characters except that one. They note that it possesses a very great resistance to desiccation. Houston and McCloy describe the enterococcus, which they isolated from many cases of Trench fever, as being very resistant to heat—up to one and one-half hours at 55°C.—but they state that it does not ferment mannitol,

although otherwise an active fermenter. Dible found a small minority of non-mannitol fermenters among heat resistant streptococci from the intestine, and regards mannitol fermentation as a secondary, although important character. He is very emphatic that diplococcal morphology correlates closely with heat resistance and mannitol fermentation, as opposed to the association of a tendency to long chain formation with heat sensitiveness and failure to ferment mannitol. As noted above, I find from the observations on the present series that the morphological criterion is not so decisive as he seems to consider,—chiefly since many heat sensitive, non-mannitol-fermenting strains appear as rather oval cocci in pairs or short chains, while not all heat resistant forms are typically lanceolate diplococci. Bagger found that 100 per cent of 150 strains of enterococci isolated from the intestine fermented mannitol.

In regard to the classification of the heat sensitive strains, the large majority of these (30 out of 35) would be classed as *Streptococcus mitis* according to Holman's method, by reason of non-hemolysis, fermentation of lactose and salicin and no fermentation of mannitol. Those of the *mitis* strains which came from the intestine or the feces grew well on McConkey's medium, while similar streptococci from the mouth did not. Dible found likewise that the majority of non-heat-resistant forms were of the type of *Streptococcus mitis*.

Two strains of *Streptococcus salivarius* (nos. 44 and 45) were isolated from the duodenum and mouth of the same man. These did not grow on McConkey's medium, and were definitely long-chained.

A *Streptococcus non-hemolyticus* III (no. 51) was isolated from the stomach of a dog.

In 6 strains from the feces or intestines of rats, all were lanceolate diplococci of the morphological type III. Five of them grew on McConkey's medium and 4 of these were heat resistant and mannitol fermenting, the fifth being neither heat resistant nor mannitol fermenting. The sixth strain possessed none of the three qualities mentioned.

In conclusion, a few words may be said with regard to the sig-

nificance attached to the name enterococcus and the justification for using it to distinguish a definite group of organisms. It has already been noted that Thiercelin used the name to designate a pleomorphic coccus which was sensitive to heat, not capable of fermenting sugars and toxic to mice, but the name is now associated by the work of several investigators (Houston and McCloy, Dible, Bagger) with a heat-resistant coccus, an active fermenter, relatively non-toxic to mice. The uniformity of description among these writers and the similarity of the organisms which they describe to the heat-resistant strains found in the present study seem to justify the adoption of a specific name for this heat-resistant group of paired or chained cocci; but it must be remembered that, as stated by Dible and corroborated above, the heat resistance test is not an absolute one, and that among the other characteristics of the organism morphology and mannitol fermentation are not invariable in the results of all workers. On the other hand, low toxicity to animals is found in all cases by those who tested it, and growth on bile-salt medium was found in all cases in my series, which were derived, however, only from the alimentary canal.

The separation of a heat-resistant group of streptococci appears justifiable, therefore, and the variation of certain individuals, in regard to one or more characteristics, from the condition of the majority is wholly in line with the knowledge of all other streptococci and other bacteria as to inter-relation of types and variation of characters in a single strain from time to time.

It must be admitted that the incompleteness of the growing knowledge of variation or mutation of organisms compels us to consider the naming of sub-species as probably of temporary value. With that in mind, it seems justified at present to retain the name enterococcus for the heat-resistant paired or short-chained cocci.

#### CONCLUSIONS

We believe that our work confirms the opinion that there is a clearly defined group of organisms sufficiently differentiated to be classified together as enterococci. Like many other bacteria,

there are atypical varieties which are intermediate between the typical ones and closely related varieties.

We would specify the attributes which justify the inclusion of an organism in the enterococcus group as follows: (1) cocci tending to be oval in shape and occurring in pairs or short chains, (2) heat resistant up to 60° for ten minutes, and (3) non-hemolytic and capable of fermenting mannitol, as secondary and not invariable characters.

Among 51 strains of streptococci isolated from the alimentary tract of man, dog and rat, 16 (31 per cent) conformed to the description of enterococci (Dible).

The remainder of the streptococci isolated from feces or intestine, with one exception, grew on a bile-salt medium and were non-mannitol fermenters and heat sensitive, and showed the sugar reactions of *Streptococcus mitis* (Holman).

Six streptococci obtained from the mouth or stomach and one obtained from the duodenum did not grow on a bile-salt medium and were of the type of *Streptococcus mitis*, *S. salivarius* or *S. non-hemolyticus* III (Holman).

It is a great pleasure to thank Dr. L. S. P. Davidson for giving me the primary cultures used in this work, and Professor T. J. Mackie for his constant assistance and interest.

#### REFERENCES

- ANDREWES, F. W., AND HORDER, T. J. 1906 *Lancet*, **2**, 708.  
 BAGGER, S. V. 1926 *Jour. Path.*, **29**, 225.  
 DIBLE, J. H. 1921 *Jour. Path.*, **24**, 3.  
 GORDON, M. H. 1905 *Lancet*, **2**, 1400.  
 HOLMAN, W. L. 1916 *Jour. Med. Res.*, **34**, 377.  
 HOUSTON, A. C. 1903-1904 *Local Govt. Board Reports*, **33**, 388.  
 HOUSTON, T., AND McCLOY, J. M. 1916 *Lancet*, **2**, 632.  
 KOPELOFF, N., AND BEERMAN, P. 1922-1923 *Proc. Soc. Exp. Biol. and Med.*, **20**, 71.  
 THIERCELIN 1899 *Compt. Rend. Soc. Biol.*, **51**, 269, 551.  
 THIERCELIN 1902 *Ibid.*, **54**, 1082.



# THE INFLUENCE OF IODINE UPON THE GROWTH AND METABOLISM OF YEASTS<sup>1</sup>

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There are certain elements which are required in comparatively large quantities for the normal growth and reproduction of organisms. Other elements are required in smaller quantities whereas still others, while not essential, if supplied modify and often accelerate growth and metabolism. That our knowledge of the required elements is less complete concerning the microscopic forms of life than it is concerning the macroscopic forms is appreciated by all who have tried to grow yeast and some bacteria upon a strictly known mineral salt-sugar solution. Under such conditions growth is often absent or meagre in quantity, or metabolic activity is perverted. This was forcefully brought to our attention during the past year in a study that was made of new species of nitrogen-fixing microorganisms obtained from a typical arid soil. Some microorganisms were obtained which are energetic nitrogen fixers if grown in soil or soil extract media containing an appropriate source of energy, but, so far, all efforts to obtain fixation upon a known mineral salt-sugar medium have failed. This led to the investigation of the influence of certain substances (which occur in plants, soils, and waters in minute quantities) upon the growth and metabolism of microorganisms. Preliminary experiments have been performed on yeasts, as they lend themselves more readily to growth and metabolic experiments than do the nitrogen-fixing bacteria. The direct microscopic count of the number of cells at varying times was taken as a measure of growth

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and the carbon dioxide evolved as a measure of metabolism. In this paper are given the results obtained when yeasts are grown in the presence and absence of iodine, in a mineral salt-sugar medium.

#### METHODS OF EXPERIMENTATION

The early work was with commercial yeasts, and later work with pure cultures. It was necessary to devise means for insuring the continuous purity of the cultures throughout the progress of each specific test. Considerable difficulty was experienced in accomplishing this and at the same time assuring an adequate supply of oxygen to the cultures. The yeasts were cultured in synthetic media in specially arranged Erlenmeyer flasks. At intervals the number of yeasts in the cultures and the quantity of carbon dioxide evolved was determined.

#### *Media*

Definite information concerning nutrient requirements of microorganisms has come from studies in which known cultural media are used; i.e., those prepared from pure chemicals, the composition of which is known. After considerable preliminary work with various synthetic media, Mayer's culture fluid was found best suited as the carrier of the iodine. Its composition is as follows:

Sugar .....	15 0 grams
Potassium phosphate (monobasic) .....	5 0 grams
Magnesium sulfate.....	5.0 grams
Calcium phosphate (dibasic) .....	0.5 gram
Ammonium nitrate.....	0.75 gram
Distilled water.. .....	1000.00 cc.

The inorganic constituents were Baker's highest purity. The water was carefully distilled. This medium was used as the carrier of varying quantities and kinds of iodine which were added from carefully standardized solutions prepared from salts of the highest obtainable purity. In the early work, commercial beet sugar was used as the source of carbon. Later, Baker's highest purity glucose and sucrose which had been carefully washed with

80 per cent hot alcohol was employed. Consequently, the composition of each specific cultural medium is quite accurately known.

### *Cultural method*

The yeasts were cultured in 500-cc. Erlenmeyer flasks fitted with 3-hole rubber stoppers. Through the first hole passed a 3-inch piece of glass tubing having a  $\frac{3}{8}$ -inch bore and closed at the top with a rubber stopper. Over this was inverted a snugly fitting test tube. Samples were removed through this opening with sterile pipettes, as required for the making of counts. The second hole contained a tube bent at 90°, the end of which entered the flask dipping into the culture fluid. The end was drawn out to a fine point. This served as an inlet for the sterile carbon-dioxide-free-air and insured adequate aeration, which is so essential for the rapid multiplication of yeasts. In the earlier work, air was drawn through cotton and soda lime to free it of microorganisms and carbon dioxide, but, it soon became evident that if tests were to be conducted over long periods microorganisms would be drawn through the cotton. Consequently, in the later tests, air was drawn through bottles containing in the order named, sulfuric acid, potassium hydroxide, tartaric acid and finally sterile distilled water. The third hole in the stopper of the cultural flask contained a bent tube which passed just through the stopper and thence to a second 500-cc. Erlenmeyer flask containing  $N/5$  barium hydroxide. This flask was fitted with a 2-hole stopper, the second opening of which contained a tube leading to an exhaust pump. When the pump was in action sterile air, free from carbon dioxide, was drawn into the culture fluid and the evolved carbon dioxide was drawn into the barium hydroxide, which was carefully protected against outside contamination. The culture flasks were immersed in a water bath kept at 28° to 30°C. by means of an electrical heating device.

In the early experiments the flasks were inoculated from a suspension of yeasts. This was prepared by triturating 1 gram of yeast cake (Fleischman's or Red Star) in 100 cc. of Mayer's culture fluid until the yeasts were evenly distributed. In later work



a loopful of yeast from a known culture was placed into 100 cc. of Mayer's culture fluid and then shaken until the yeasts were uniformly distributed. Measured portions of these suspensions were used for the seeding of the cultural flasks.

The carbon dioxide was determined by collecting in  $N/5$  barium hydroxide and titrating with standard oxalic acid using phenolphthalein as the indicator. The results are reported as milligrams of carbon dioxide produced up to the specified times. At the same time the number of yeasts in the culture fluid was determined by the removal of one cubic centimeter portions using aseptic precautions so as not to contaminate the cultures. Counts were made by diluting to the required extent and then placing a drop on the disk of a hemocytometer. Ten groups of 25 squares were counted; in the absence of agreement among duplicates this was repeated. The reported results are the average of a number of determinations and represent the number of yeasts found in one cubic millimeter of the culture solution at the specified time. One of the greatest obstacles encountered in the work was the obtaining of representative samples, as the yeasts tend to adhere. The constant drawing of air through the culture obviated this difficulty only to a degree.

#### COMMERCIAL YEASTS WITH SODIUM IODIDE

The first tests were made on commercial yeasts—Fleischman's in some and Red Star in others. One gram portions of the yeast cake were triturated with 500 grams of sterile distilled water. One cc. portions of this suspension were distributed to the various flasks, each containing 100 cc. of Mayer's culture solution with commercial beet sugar as the source of carbon. The initial inoculation in the various sets, the averages of which are reported in tables 1 and 2 varied from 5000 to 10,000 yeast cells. The iodine content of the media varied from 0 to 8000 parts per million. The number of yeasts and the quantity of carbon dioxide produced were determined at intervals. The averages for four such sets of determinations are given in tables 1 and 2.

Earlier tests had shown that when large quantities of the inoculum were used rapid growth occurred in all the cultures. It is

evident from these results that the initial inoculum must have been nearly the same in the various flasks and that growth in Mayer's culture fluid is extremely slow. Growth is accelerated when one part per million of iodine in the form of sodium iodide is added to the culture medium. The maximum stimulation is produced when 10 parts per million is present. In this iodine concentration the yeast cells at the end of thirty seven hours are four times as numerous as they are in the straight Mayer's medium. In the higher concentrations of iodine there is a longer lag

TABLE 1

*Number of yeasts per cubic millimeter produced in Mayer's culture fluid containing varying quantities of iodine as sodium iodide with commercial beet sugar as the source of carbon*

IODINE AS NaI	THOUSANDS PRODUCED DURING							
	24 hours	26 hours	28 hours	30 hours	32 hours	34 hours	36 hours	37 hours
<i>p. p. m.</i>								
None	5	5	5	6	6	7	7	8
1	5	5	6	8	8	11	12	16
10	5	8	12	12	15	25	25	31
100	5	5	5	6	7	19	16	22
1,000	5	5	7	7	10	26	18	30
2,000	4	4	5	9	14	20	18	25
3,000	4	5	6	7	7	14	15	18
4,000	5	5	5	5	5	14	13	15
5,000	3	4	5	5	5	13	11	15
6,000	3	5	4	8	10	21	35	39
7,000	2	2	3	4	4	8	8	9
8,000	4	4	4	3	4	20	15	22

period, probably due to the yeast becoming acclimated to the iodine-containing medium. There is no evidence of toxicity in any of the concentrations tested, and the results point to a definite stimulation, even with the minute quantity of one part per million.

At the same time that counts were made, the quantities of carbon dioxide which had been evolved were determined. The averages, stated as milligrams of carbon dioxide produced in the various intervals, are given in table 2.

There was a gradual increase from period to period in the carbon dioxide produced. This is not what would be anticipated from the action of a definite number of cells and indicates either increased number of producing cells or increased efficiency. It would seem to be due to multiplication and not increased metabolism. Iodine stimulates even in one part per million and reaches its maximum efficiency in a concentration of 10 parts per million. The low results obtained when 7000 parts per million of iodine were present would be accounted for by errors which may have

TABLE 2

*Milligrams of carbon dioxide produced by yeast in 100 cc. of Mayer's solution of culture fluid containing varying quantities of iodine as sodium iodide with commercial beet sugar as the source of carbon*

IODINE AS NaI	MG. CARBON DIOXIDE PRODUCED DURING							
	24 hours	26 hours	28 hours	30 hours	32 hours	34 hours	36 hours	37 hours
<i>p. p. m.</i>								
None	50	61	86	108	138	179	221	255
1	50	67	85	109	148	196	245	281
10	57	77	99	131	176	231	281	316
100	49	64	82	116	162	218	276	330
1,000	51	66	83	114	158	204	253	288
2,000	42	56	74	97	127	165	203	224
3,000	54	70	84	101	121	148	176	199
4,000	47	58	71	85	105	130	154	178
5,000	36	47	61	80	104	127	157	181
6,000	51	67	87	122	170	225	269	306
7,000	13	20	28	38	50	67	84	100
8,000	60	79	98	134	130	212	276	306

crept into the work, were it not for the fact that it appeared to a greater or less extent in all sets and occurred even when the various flasks were interchanged on the apparatus. When the milligrams of carbon dioxide per 1000 yeasts are calculated it is found that in the absence of iodine the production of carbon dioxide for each individual cell gradually increases throughout the reaction, i.e., it follows the law of an autocatalyzed reaction. In the presence of iodine the production of carbon dioxide is more nearly constant, indicating that the older cells are more efficient in the absence of iodine than in its presence. The results point to the conclusion

that sodium iodide increases growth but does not increase individual metabolic activity.

#### INFLUENCE OF POTASSIUM IODIDE UPON COMMERCIAL YEASTS

Commercial yeasts were also cultured in the presence of potassium iodide. The same concentrations of iodine were used as in the sodium iodide series with the same results. The iodine stimulated growth when present in the culture medium in small quantities. The quantity of carbon dioxide produced was increased; this was due to an increased number of cells and not to an increased efficiency. In short, the result were so nearly a duplicate of those obtained with the sodium iodide that only these general conclusions need be given.

#### INFLUENCE OF CALCIUM IODIDE UPON COMMERCIAL YEASTS

When calcium was used as the carrier of iodine the stimulation was also apparent. There was a decrease in the lag period but no evidence of an increased metabolic activity of individual cells. The increase in carbon dioxide came from an increase in the number of cells. The rate of multiplication and also the metabolic activity in all series were markedly influenced by the size of the inoculum, the influence of the iodine being most clearly manifested when small seedings were used.

#### INFLUENCE OF ELEMENTARY IODINE UPON COMMERCIAL YEASTS

There often appeared in the culture fluid a slightly yellowish tinge, especially in those cultural flasks exposed directly to the light. This was probably elementary iodine; hence the question arises as to whether or not elementary iodine is toxic. Furthermore, in order to make more certain that it was the iodine and not the cation, sodium, potassium, or calcium, which acted as the stimulant, a series was run in which varying quantities of elementary iodine were added to Mayer's culture fluid. A standard alcoholic solution of iodine was prepared and this was used to introduce into the culture media the required quantity of iodine. To those cultures receiving small quantities of iodine or no iodine,

there was added sufficient alcohol so that all received the same amount of alcohol, whether or not they received iodine. Consequently, the only variation in their treatment was in the quantity of elementary iodine which they received. The results are given in table 3.

A slight stimulation is noted in the presence of one part per million of elementary iodine. It is probable that smaller quanti-

TABLE 3

*Number of yeasts produced in Mayer's culture fluid containing varying quantities of iodine*

IODINE AS I <sub>2</sub>	THOUSANDS OF YEASTS IN MEDIUM AT THE END OF						
	24 hours	26 hours	28 hours	30 hours	32 hours	34 hours	36 hours
<i>p.p.m.</i>							
None	6	8	9	11	11	17	11
1	6	7	8	12	12	19	16
10	3	5	6	6	6	6	7
100	2	2	3	5	5	5	7
1,000	0.6	0.6	0.6	0.6	0.6	0.6	0.6
2,000	0.6	0.6	0.6	0.6	0.6	0.6	0.6

TABLE 4

*Milligrams of carbon dioxide produced in Mayer's culture fluid containing varying quantities of elementary iodine*

IODINE	24 HOURS	26 HOURS	28 HOURS	30 HOURS	32 HOURS	34 HOURS	36 HOURS
<i>p.p.m.</i>							
None	65	89	106	117	128	143	158
1	70	93	106	120	132	148	164
10	51	68	80	94	108	122	132
100	20	33	42	55	68	81	95
1,000	6	15	19	21	24	28	32
2,000	6	15	19	21	23	27	31

ties of elementary iodine would have to be used to obtain the full stimulating effect, as 10 parts per million act as a weak antiseptic; and when 1000 parts per million are present all growth is prevented. It is evident that when one part per million of iodine in the form of the various salts is added to the culture media there would never be sufficient elementary iodine liberated to prove toxic to the yeast. With larger quantities this may not be true,

and it is quite possible that the erratic results which so often appeared have been due to this factor. The quantities of carbon dioxide produced under the same conditions are given in table 4.

One part per million of elementary iodine slightly increases carbon dioxide production, corresponding to the increased number of cells. Higher concentrations are toxic. The iodine retards growth to a greater extent than it does metabolism.

The results with commercial cultures of yeasts warrant the conclusions that various salts of iodine, together with elementary iodine when added to Mayer's cultural fluid, stimulate growth of yeasts. The greatest stimulation, with the concentrations tested,

TABLE 5

*Number of yeast cells produced in Mayer's solution with and without iodine and in the presence of various carbohydrates*

TREATMENT (I AS KI)	THOUSANDS OF YEASTS PRODUCED IN							
	24 hours	30 hours	34 hours	50 hours	98 hours	146 hours	194 hours	218 hours
Sucrose .. . . . . .	3	9	10	25	72	155	158	158
Sucrose + 100 p.p.m. I.....	5	10	13	56	76	196	175	195
Lactose .. . . . . .	1	1	1	3	2	3	3	3
Lactose + 100 p.p.m. I.....	1	1	1	2	3	3	3	3
Maltose. .... .	1	2	2	3	26	53	101	158
Maltose + 100 p.p.m. I. ....	2	3	3	5	23	46	95	98

was when one part per million of iodine is present. Whether even smaller quantities will stimulate cannot be answered from these results.

#### INFLUENCE OF IODINE IN THE PRESENCE OF VARIOUS CARBOHYDRATES

A series was run in which various carbohydrates were used as the source of carbon, with and without potassium iodide. Unfortunately, one part of iodine was used in 10,000 parts of the media. The results as to numbers are reported in table 5.

Good growth occurred in the presence of sucrose and maltose,

but little occurred in lactose, the yeast being unable to assimilate it. Growth was increased in the sucrose containing iodine, but no effect was produced by iodine in the presence of maltose or lactose in the concentrations of iodine tested. Would it have stimulated had the concentrations been only one part per million?

The quantities of carbon dioxide evolved by yeasts in the presence of iodine and with various carbohydrates as the source of energy are given in table 6.

The increased carbon dioxide resulting from the use of the iodine in the presence of sucrose is in keeping with the results already reported. That is, the carbon dioxide increases with greater

TABLE 6

*Milligrams of carbon dioxide produced by yeasts in Mayer's culture fluid with and without iodine with various carbohydrates as the source of energy*

TREATMENT (I AS KI)	24 HOURS	30 HOURS	34 HOURS	50 HOURS	93 HOURS	141 HOURS	194 HOURS	218 HOURS
Sucrose .....	19	50	91	374	609	713	770	796
Sucrose + 100 p.p.m. I. ....	21	55	99	344	586	684	787	807
Lactose .....	9	22	29	60	108	120	133	152
Lactose + 100 p.p.m. I. ....	8	20	26	66	118	127	139	151
Maltose .....	11	27	38	114	346	566	633	676
Maltose + 100 p.p.m. I. ....	13	31	41	119	351	580	665	728

number of cells resulting from the stimulation of growth by the iodine. Although the yeasts did not readily multiply in the presence of lactose, yet their metabolic activities were comparatively active, pointing to the conclusion that metabolic activity is increased by iodine in the presence of maltose, and especially lactose.

#### INFLUENCE OF IODINE ON PURE CULTURES OF YEASTS

The results so far reported were obtained with commercial cultures of yeasts. Therefore, the questions arise: Can they be duplicated with pure cultures: Are they characteristic of yeasts or, are they secondary effects from the action of iodine upon other microorganisms and do these in turn influence the growth and

metabolism of the yeasts? There is also the possibility of substances occurring in the commercial beet sugar or in the minute seeding carried over in the inoculum which may be acted upon by iodine so that the resulting compound will act as a stimulant. For these reasons, cultures of yeasts were obtained from Dr. F. W. Tanner of the University of Illinois. These were grown in cultural media of known composition. Greater precautions were taken to prevent contamination from outside sources, and more

TABLE 7

*Yeast cells (Saccharomyces cerevisiae) produced in Mayer's solution containing varying quantities of potassium iodide with commercial beet sugar as the source of carbon*

IODINE AS KI	THOUSANDS OF CELLS AFTER					
	50 hours	74 hours	98 hours	122 hours	146 hours	170 hours
<i>p. p. m.</i>						
None	0 3	1.0	6	6	8	9
1	0.3	0 3	3	3	6	13
10	0.3	0 5	2	3	35	35
100	0.3	0.3	1	2	24	28
1,000	0.3	0.4	2	22	33	52
2,000	0 3	2 0	2	21	36	57
3,000	0.3	1.0	5	5	42	46
4,000	0 3	1 0	2	2	16	19
5,000	0 3	0 5	0 6	1	10	34
6,000	0 3	0 3	1	1	24	31
7,000	0.3	1 0	3	3	17	40
8,000	0 3	4 0	10	8	32	

care used to prevent unknown constituents from being carried over in the inoculation. The size of the seeding was also quite accurately known.

#### INFLUENCE OF IODINE ON TYPES OF SACCHAROMYCES CEREVISIAE

In the first series, *Saccharomyces cerevisiae* were cultured in Mayer's culture fluid containing various quantities of potassium iodide with commercial beet sugar as the source of carbon. The average results for four determinations are given in tables 7 and 8.

Many workers have found, when large numbers of yeasts are



used for seeding the culture media, that growth is better than when small numbers are used, and that if the seeding into synthetic media be extremely small, either no growth or very meagre growth occurs. Consequently, special precautions were taken to insure uniform seeding and to possess actual knowledge concerning the size of the seeding. This was soon accomplished by suspending a loopful of the culture in 100 cc. Mayer's culture fluid. Counts were made on this; it was then diluted so that each cubic centimeter contained approximately 100 cells. One cubic centimeter portion of this suspension was seeded into each cul-

TABLE 8

*Milligrams of carbon dioxide produced by yeasts (Saccharomyces cerevisiae) in the presence of varying quantities of potassium iodide with commercial beet sugar as the source of carbon*

IODINE AS KI	24 HOURS	50 HOURS	74 HOURS	98 HOURS	122 HOURS	146 HOURS	170 HOURS
<i>p.p.m.</i>							
None	4	17	18	116	323	372	436
1	4	8	20	159	313	363	446
10	6	17	26	71	261	380	428
100	5	22	37	112	258	390	495
1,000	5	17	36	172	364	449	514
2,000	11	22	37	165	389	469	523
3,000	5	27	40	119	279	397	460
4,000	5	18	31	106	156	308	393
5,000	6	18	31	97	144	207	371
6,000	5	16	21	70	187	301	370
7,000	5	18	31	121	258	344	493
8,000	4	19	35	115	188	264	358

tural flask. That this was quite uniform and that all flasks contained living yeasts is evident from the fact that the concentration of yeast at the end of twenty-four hours was over 320 cells per cubic millimeter. It is also evident that the results with pure cultures are not far different from those obtained with commercial cultures. Growth is extremely slow and is stimulated by small quantities of iodine. The spread of the concentration of iodine which stimulates is greater than with the commercial yeasts, and there is no concentration which can be considered toxic. The average results for the metabolism experiments are reported in table 8.

Considerable carbon dioxide was produced in this medium, thus indicating that it contained sufficient bios for growth and metabolism, even though the seeding was small.

The results give evidence of a shortened lag period due to the iodine. The production of carbon dioxide has been increased due to the iodine, and although it does not occur at the same concentration as was found in the commercial cultures yet it occurs when only small quantities of iodine are added. The stimulation becomes more evident as the time of the growth is prolonged. By the end of 194 hours it was very evident, even at a concentration of one part per million of iodine. However, these results were discarded and the experiment terminated as there was evidence in some of the cultural flasks of contamination. It has been our repeated observation that when bacteria, and especially mold growths, appear in cultural flasks, the results are very materially changed. Under such conditions even in the non-iodine containing cultures there was an increased growth and metabolism of the yeasts.

The results with the pure cultures are quite similar to those obtained with the commercial yeasts. When the pure cultures of *Saccharomyces cerevisiae* were grown with sodium iodide in varying concentrations of lactose or maltose the results confirmed those obtained with the commercial yeasts; consequently, the results are not reported here.

The results so far obtained have demonstrated the following: (1) Various salts of iodine increases growth and metabolism of the yeast cells. Apparently multiplication is increased more in proportion than is respiration. (2) Sufficient bios is present in commercial beet sugar to furnish the requisite accessory food factors even when highly purified salts are used as the basis of the nutrient medium. (3) Yeast will readily grow in Mayer's nutrient medium if large quantities of yeast are used to make the inoculation.

#### PURIFIED CARBOHYDRATES

In an attempt to substitute iodine for bios, yeasts were cultured in the presence of varying quantities of iodine, using Baker's

highest purity glucose as the source of carbon. The glucose was substituted for the sucrose as the latter is not readily fermented by the yeasts used. Mayer's culture fluid was prepared from Baker's highest purity chemicals. The stock culture of yeasts (*Saccharomyces cerevisiae*) was inoculated into 100 cc. of this medium and microscopic counts made of the number of cells in 1 cc. of the medium. It was then diluted to such an extent that each cubic centimeter contained 50 yeasts. One cubic centimeter portion of this solution was distributed to each of the culture media with varying quantities of potassium iodide. At intervals, counts and carbon dioxide determinations were made. The numbers of organisms found in one cubic millimeter are reported in table 9.

TABLE 9  
*Number of yeasts (Saccharomyces cerevisiae) in Mayer's solution containing varying quantities of potassium iodide and highly purified glucose*

IODINE AS KI	74 HOURS	98 HOURS	122 HOURS	146 HOURS	170 HOURS	194 HOURS	218 HOURS	242 HOURS	266 HOURS	290 HOURS	314 HOURS	318 HOURS
<i>p. p. m.</i>												
None	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	0 6	0 4	19	33	33	35	47	49	10	17
10	+	+	+	+	0 6	3	42	65	13	14	14	14
100	+	0 6	0 6	0 6	0.6	0 6	0 6	0.6	2	5	52	61
1,000	+	+	+	+	+	+	+	+	+	3	52	66
2,000	+	+	+	+	+	+	+	+	+	+	+	8
3,000	+	+	+	+	+	+	+	+	+	+	+	+

These represent the average results of three separate determinations.

The culture media were inoculated with 50 yeasts per cubic-centimeter. This would be only one cell in each 20 c. mm., a number so small that they could not be enumerated by the microscope method with any degree of accuracy; consequently, where only one cell was found in the ruled spaces of the hemocytometer it is listed in the above table with a + sign and may indicate from 1 to 320 in a cubic millimeter of the solution. Where the yeasts were more numerous figures are given which represent the number of thousands of yeasts in 1 c. mm. of the culture fluid.

In the culture medium containing no potassium iodide there

were never over 320 yeasts per cubic millimeter. Consequently, multiplication must have been extremely slow. In the presence of 1 p.p.m. of iodine, the number had reached 19,000,000 at the end of 170 hours; at the end of 290 hours there were 49,000,000 yeasts. After 290 hours there was a decrease. The numbers are even

TABLE 10

*Milligrams of carbon dioxide produced by Saccharomyces cerevisiae in the presence of varying quantities of iodine as potassium iodide and with Baker's highest purity glucose as the source of carbon*

IODINE AS KI	24 HOURS	50 HOURS	74 HOURS	98 HOURS	122 HOURS	146 HOURS	170 HOURS	194 HOURS	218 HOURS	242 HOURS	266 HOURS	290 HOURS	314 HOURS	338 HOURS
<i>p. p. m.</i>														
None	2	3	4	34	38	41	43	43	43	45	46	56	57	88
1	2	15	16	17	20	61	200	277	314	348	376	442	480	500
10	3	5	5	6	18	22	23	37	90	131	156	190	209	236
100	4	6	7	7	11	15	18	21	28	41	57	118	172	221
1,000	2	3	5	8	12	13	15	32	44	56	60	124	205	248
2,000	2	4	4	6	42	56	60	55	66	69	77	95	126	226
3,000	2	4	6	7	13	13	14	18	18	20	20	24	33	34

TABLE 11

*Number of yeasts (Saccharomyces cerevisiae) in Mayer's solution containing varying quantities of potassium iodide and highly purified sucrose*

IODINE AS KI	96 HOURS	120 HOURS	144 HOURS	168 HOURS	192 HOURS	216 HOURS	240 HOURS	264 HOURS	288 HOURS	312 HOURS	336 HOURS
<i>p p m.</i>											
None	+	+	+	+	+	+	+	1	1	1	6
1	+	+	+	+	+	+	+	4	26	38	37
10	+	+	+	+	+	+	+	+	1	2	2
1,000	+	+	+	+	+	+	+	1	17	22	23
2,000	+	+	+	+	+	+	+	+	+	2	6
3,000	+	+	+	+	+	+	+	+	+	1	2

greater in the presence of 10 p.p.m. of iodine. At 100 and 1000 p.p.m. the lag period is lengthened over what it is in the lower concentrations; apparently the decline does not set in as early. In 2000 p.p.m. of iodine multiplication is very slow and in 3000 p.p.m. it is not perceptible.

A very small, constant quantity of carbon dioxide is evolved in the absence of iodine but in the presence of one part per million of iodine there is an increase in the evolution of carbon dioxide in keeping with the increased number of yeast cells. In the presence of higher concentration there is also an increase of carbon dioxide even up to 2000 parts per million. Above this concentration the iodine apparently is toxic for in the presence of 3000 parts per million of iodine there is no apparent increase in yeast cells and a decrease in the carbon dioxide evolved.

In an attempt to determine if iodine stimulates when carefully purified sucrose is used as a source of carbon, a similar set was

TABLE 12

*Milligrams of carbon dioxide produced by Saccharomyces cerevisiae in the presence of varying quantities of iodine as potassium iodide and with purified sucrose as the source of carbon*

IODINE AS KI	24 HOURS	48 HOURS	72 HOURS	96 HOURS	120 HOURS	144 HOURS	168 HOURS	192 HOURS	216 HOURS	240 HOURS	264 HOURS	288 HOURS	312 HOURS	336 HOURS
<i>p.p.m.</i>														
None	1	3	5	6	7	8	9	9	11	13	13	16	30	83
1	1	3	3	4	6	13	19	25	28	29	53	173	220	236
10	2	2	9	23	30	37	44	50	55	63	86	114	145	175
1,000	6	24	47	75	100	114	139	169	213	237	244	289	387	409
2,000	4	8	17	18	28	46	58	69	77	101	132	147	158	181
3,000	6	8	9	11	11	18	32	48	60	62	70	85	92	101

run through using sucrose as the carbohydrate. Commercial beet sugar was purified by repeated washing with 80 per cent hot ethyl alcohol. This purified sucrose was used in the making of Mayer's nutrient medium. Baker's highest purity chemicals were used throughout and everything with the exception of the carbohydrate, kept as nearly comparable with the preceding series as possible. Each cultural flask was inoculated with a suspension of yeast in sufficient quantities to add 50 yeast cells to each cubic centimeter of the cultural medium. These were then incubated at 28°C. and at intervals determinations were made of the number of yeasts and milligrams of carbon dioxide evolved. The average results for numbers are given in table 11.

The small initial inoculation accounts for the extremely long time which elapsed before growth was perceptible, but the fact that yeasts were always found on microscopic examination proved that all flasks were inoculated. Even one part per million of iodine stimulated growth. These results are almost an exact duplicate of those obtained when this type of yeast was grown with purified glucose as the source of carbon, thus making it certain that the organism is stimulated by iodine or that iodine can substitute for the bios.

At the same time that counts were made the carbon dioxide evolved was determined. The averages for these results are given in table 12.

The results for the metabolism indicate that no appreciable multiplication of the yeast cells occurred in the iodine-free medium, as the quantity of carbon dioxide produced in unit time is a constant. However, in the presence of iodine it increases. This is very noticeable even in the presence of 1 p.p.m. of iodine, but like growth, metabolism reaches its maximum in the presence of 1000 p.p.m. of iodine. It is evident that both multiplication and respiration are influenced by iodine, whether the source of carbon be purified glucose or purified sucrose.

Other series were run in which all conditions were kept as nearly as possible the same as in the above series with the exception of the yeast. In these series, *Saccharomyces cerevisiae* Type Frobergh and *Saccharomyces cerevisiae* were used. The results were similar to those reported.

It required 288 hours in the synthetic media devoid of iodine for the yeasts to become numerous enough to enumerate by the microscopic method. During the following forty-eight hours the yeast made consistent gains in this medium. In similar media containing 1 p.p.m. of iodine as potassium iodide the yeasts were sufficiently numerous to enumerate after 144 hours; by the end of 336 hours there were 102,000 yeasts in each cubic millimeter. In higher concentrations there was also a stimulation, but it was neither as great nor as uniform as in the lower concentrations of iodine. There was unmistakable evidence of stimulation with 1 p.p.m. of iodine which corresponded with the increase in number

of yeast cells and is in keeping with the results obtained in the other reported tests.

In all of the series there was conclusive evidence that iodine increases multiplication, even when added to the nutrient medium in a concentration of one part per million.

*Saccharomyces cerevisiae* Type Saaz, growing in Mayer's nutrient medium with carefully purified sucrose as the source of carbon, produced only 20 mgm. of carbon dioxide, whereas with some concentrations of iodine there was produced over 25 times this amount. The rate per hour of carbon dioxide production in the absence of iodine was nearly constant throughout the series, indicating little, if any, multiplication, whereas in the presence of iodine it was progressively increased, as is characteristic of an autocatalyzed chemical reaction.

It was often desired to conduct the experiment for a longer period, but usually the work was terminated through the fact that molds or less often bacteria found their way into the culture solution. In the work so far reported, cotton and large tubes of soda lime were depended upon to keep out microorganisms and carbon dioxide. In order to make it possible to conduct the tests for a longer period and to find whether these yeasts multiply when inoculated into synthetic media in very small numbers, and to determine whether the stimulation by iodine has only a temporary effect or will last over long periods, a series was run with the following modifications: (1) The air was drawn through strong acid and alkali and then through cotton and soda lime. All connections were sealed with wax, thus making it possible to conduct the experiment over long periods without contamination of the cultural solutions. (2) Baker's highest purity sucrose was carefully washed with hot 80 per cent alcohol and this sugar used as the source of carbon. (3) The cultures were seeded with only 150 yeasts to the flask. It is claimed by some workers that a pure synthetic medium seeded very lightly does not promote growth, while others maintain that it does; however, the growth is extremely slow. The average results for three such sets are given in table 13.

Although the results do not show an actual multiplication in the

medium containing no iodine, yet it was evident that there had been a slight multiplication as the cells were more easily found toward the close of the experiment than they were at the beginning. However, growth was very slow, as is illustrated by the fact that even after thirty-one days there were not over 320 cells to the cubic millimeter. When iodine was supplied, multiplication was rapid enough so that toward the close of the experiment in some of the concentrations of iodine they reached thousands in 1 c. mm. However, even in the presence of iodine there is only a slow growth. The quantities of carbon dioxide produced under these conditions are given in table 14.

TABLE 13

*Number of yeasts (Saccharomyces cerevisiae) produced in Mayer's nutrient solution with and without iodine and with highly purified sucrose as the source of carbon*

IODINE AS KI	9 DAYS	11 DAYS	13 DAYS	15 DAYS	17 DAYS	19 DAYS	21 DAYS	23 DAYS	25 DAYS	27 DAYS	29 DAYS	31 DAYS
<i>p.p.m.</i>												
None	—	+	+	+	+	+	+	+	+	+	+	+
1	—	+	+	+	+	+	+	+	+	+	1	1
10	2	2	4	6	7	7	6	5	7	6	10	11
100	—	+	+	3	5	8	6	8	12	12	13	16
1,000	2	3	4	4	4	7	8	8	9	9	11	11
2,000	—	+	+	+	+	1	1	1	2	2	2	3
3,000	—	+	+	+	+	+	1	1	1	1	1	1
4,000	—	+	+	+	+	+	+	+	+	+	1	1

At the beginning of the experiment the non-iodine-containing series was producing carbon dioxide at the rate of 1 mgm. per twenty-four hours. This regularly increased, so that by the end of the experiment it was being produced at the rate of 7 mgm. per twenty-four hours. If there was no change in efficiency there would have been 7-fold multiplication in thirty-one days. This is extremely slow. By the same reasoning it may be concluded that in the presence of 100 p.p.m. of iodine there had been three times this growth. Counts, however, indicate that there had been an even greater increase than this.



## GENERAL CONCLUSIONS

It is evident from these results that the growth and metabolic activities of yeast are extremely slow in Mayer's cultural fluid when it is prepared from carefully purified chemicals. It is also evident that impure chemicals, especially the carbohydrates, may contain sufficient of Wildier's bios growth-promoter, to increase growth appreciably. The same may be the case when large quantities of the yeasts are used for seeding the cultural flasks, presumably due to the carrying of sufficient bios into the new cultures. The results reported in this paper conclusively prove that minute quantities of iodine when added to an appropriate synthetic medium promote the growth of yeasts and raise a number of interesting questions:

1. What is the relationship of Wildier's bios to iodine? If Wildier's, or later Develo's,<sup>2</sup> description of bios be taken as a criterion of properties, it cannot be stated that iodine meets fully the requirements of bios. Some of the properties attributed to bios could not be attributed to iodine. Moreover, the increase in growth attributed to bios is often much greater than we have been able to produce with iodine. These objections, however, do not preclude the possibility of organic or inorganic compounds of iodine being the possible cause of the phenomenon attributed to bios. There is also the possibility of iodine not being the only element required by yeast in minute quantities. It does not appear that the hypothetical bios could have been carried into these cultures by iodine and that it and not the iodine is the stimulator of growth, for: (a) Chemicals of high purity were used as the carriers of iodine; (b) sodium, potassium, and calcium iodide and also elementary iodine all stimulate and it would have to be assumed that all carried bios; (c) iodine stimulates when present in only 1 p.p.m. and possibly in even smaller concentrations. Hence, if it be concluded that the iodine is the carrier of

<sup>2</sup> Inasmuch as the whole bios question has been ably reviewed by Fred W. Tanner in the *Physiological Review* (vol. 1, no. 4, pp. 397-472), no references to the literature are given here. However, those interested are referred to Dr. Tanner's article.

impurities which are the cause of the increased growth, it would be necessary for them to be effective in almost infinitesimal quantities.

2. The absence of measurable quantities of yeast growth in the absence of iodine and the appreciable growth in its presence raises the question: Is not iodine in minute quantities required for the growth of yeast? These results indicate that it is. Is this iodine which is assimilated by the yeasts built into organic compounds which may be valuable to higher animals? If so, may this not be one of the best methods yet devised for the administration of iodine to man? It would probably be less irritating

TABLE 14

*Milligrams of carbon dioxide produced by yeast (Saccharomyces cerevisiae) when grown in Mayer's solution with and without iodine*

IODINE AS KI	7 DAYS	8 DAYS	9 DAYS	11 DAYS	13 DAYS	15 DAYS	17 DAYS	19 DAYS	21 DAYS	23 DAYS	25 DAYS	27 DAYS	29 DAYS	31 DAYS
<i>p.p.m.</i>														
None	6	9	15	26	40	56	70	83	121	145	169	200	212	225
1	3	7	14	28	47	71	89	118	138	156	172	192	206	221
10	3	7	12	23	42	64	87	120	164	205	259	306	348	390
100	4	10	13	28	42	70	107	157	228	311	387	464	534	633
1,000	7	15	22	42	99	142	235	313	394	458	494	531	557	590
2,000	4	9	15	28	45	70	93	115	141	166	187	212	235	253
3,000	3	6	11	22	39	65	89	117	142	155	172	186	200	211
4,000	4	9	18	31	50	74	109	140	175	200	235	280	313	356

than the inorganic salts of iodine and the distribution, and, consequently, dosage could be nicely controlled. Later work must answer these questions.

#### SUMMARY

Growth and metabolism of yeasts are extremely slow in a mineral salt-sugar solution such as Mayer's cultural fluid. Heavy seeding, the presence of impurities such as may occur in commercial beet sugar, and bacterial (and especially mold) growth accelerate yeast multiplication.

Small quantities of iodine, 1 p.p.m. either as elementary iodine

or as the salts of sodium, potassium, or calcium accelerate yeast growth.

The relationship of iodine to Wildier's bios and the possibility of the use of iodine-cultured yeasts as a means of furnishing iodine to man is considered. The results indicate that iodine is essential to yeast growth and metabolism.

# AN ATTEMPT TO CULTIVATE BACTERIA FROM RABBIT ENCEPHALITIS VIRUSES

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It is generally accepted that epidemic encephalitis in man and herpetic encephalitis in rabbits are not caused by the ordinary visible bacteria. The conception, however, advocated by Evans and Freeman (1926) that bacteria of ordinary size may occur in filtrable forms in these diseases demands consideration. Evans and Freeman report the isolation of a pleomorphic streptococcus from a number of human encephalitic cases, and Evans (1927) has isolated a streptococcus and spore-bearing bacteria from several rabbit encephalitic viruses, using both the unfiltered and the filtered brain emulsions. When injected into normal rabbits, these strains of streptococci gave rise to encephalitis similar to the true virus disease.

This suggestion of Evans and Freeman that a filtrable invisible virus may change into an ordinary visible form seemed of such importance that it was decided to attempt to confirm their findings.

Four strains of encephalitic virus were worked with, three strains kindly sent to us by Dr. Evans—virus C.D. (Dishman virus), virus V.D. (Devlin virus) virus E.L.<sub>1</sub> (Perdrau virus)—and one of our own strains isolated from a case of herpes. All of these gave rise to the typical rabbit encephalitis when injected intracerebrally into rabbits.

In attempting to isolate microorganisms from these viruses, the procedure of Evans and Freeman was followed as closely as possible. Cooked meat medium was prepared in accordance with the technique described by them, and this medium was

TABLE 1

VIRUS	RABBIT NUM- BER	TIME OF AUTOPSY	SMEAR EXAMINATION	CULTURE RESULTS
Herpes.....	1	Immediately after death	Negative	Staphylococcus (3 out of 8 tubes)
	2	Killed while dying	Negative	Staphylococcus (1 tube out of 9) fil- trates negative.
	3	Killed while dying	Negative	Negative
	4	Killed while dying	Negative	Negative
	5	22 hours after death	Negative	<i>B. coli</i>
	6	Glycerinated ma- terial		Negative
	7	Immediately after death	Negative	Negative
	8	Died in the night	Negative	Gram + bacillus (1 out of 9 tubes)
Perdrau.....	9	Killed	Negative	Negative
	10	Soon after death	Negative	Negative
	11	Soon after death	Negative	Negative
	12	Soon after death	Negative	Negative
	13	Soon after death	Negative	Gram + rod (1 out of 8 tubes)
			Negative	Negative
	14	Immediately after death	Negative	Negative
	15	Immediately after death	Negative	Negative
	16	Died in the night	Negative	Negative
	17	Immediately after death	Negative	Negative
	18	Immediately after death	Negative	Negative
	19	Glycerinated ma- terial		Negative
	20	Died in the night	Negative	Staphylococcus (1 out of 9 tubes)
	21	(?)	Negative	Gram + rod (1 out of 9)
	22	Immediately after death	Negative	Negative
	23	3 hours after death	Negative	Negative
	24	Immediately after death	Negative	Negative
	25	Killed	Negative	Negative
	26	Immediately after death	Negative	Negative
V. D.....	27	Died in the night	Negative	Negative
	28	Immediately after death	Negative	Negative

TABLE 1—Continued

VIRUS	RABBIT NUM- BER	TIME OF AUTOPSY	SMEAR EXAMINATION	CULTURE RESULTS
C. D. ....	29	Glycerinated ma- material		Negative
	30	23 hours after death	Negative	<i>B. coli</i>
	31	Immediately after death	Negative	Negative
	32	Soon after death	Negative	Negative
	33	Died in the night	Negative	Negative

TABLE 2

FERDRAU VIRUS			C. D. VIRUS		
Rabbit num- ber	Death and autopsy	Results of cultures	Rab- bit num- ber	Death and autopsy	Results of cultures
34	Died	Negative	62	Died	Negative
35	Killed	Negative	63	Died	Gram + cocci, <i>Micrococcus</i> <i>cremoides</i>
36	Died	Negative			
37	Died in the night	Negative	64	Killed	Diphtheroid (1 out of 3 tubes)
38	Died	Negative	65	Died	Negative
39	Died	Negative	66	Died	<i>Streptococcus viri- dans</i> (1 out of 4 tubes)
40	Died	Negative	(The above cultures were kindly made by Dr. H. Yu of this Laboratory)		
41	Died	Negative	66	(Brain after 10 days in 50 per cent glycerol)	2 out of 20 meat tubes anaerobic diphtheroid; 1 out of 20 tubes <i>B. subtilis?</i>
42	(?)	Negative			
43	Killed	Negative	67	Died	<i>Staphylococcus</i> (2 out of 10 tubes)
44	Killed	Negative	68	Killed	Negative
45	Died	Negative	69	Died	Negative
46	Died	Negative	70	Died	Negative
47	Died	Negative	71	Killed	Negative
48	Died	Negative	72	Killed	Negative
49	Died	Strict anaerobic pleomorphic diphtheroid (1 out of 4 tubes)	73	Killed	Negative
50	Died	Negative	74	Died	Negative
51	Died	Negative	75	Died	Negative
52	(?)	Negative	76	Killed	Negative
53	Died	Negative	77	Died	Negative
54	Died	Negative	78	Died	Negative
55	(?)	Negative	79	Died	Negative
56	Died	Negative	80	Died	Negative
57	Died	Negative			
58	Died	Negative			
59	Died	Negative			
60	Died	Negative			
61	Died	<i>B. coli</i>			

checked—it was found usually to be pH 6.8 or 7.0—and tested by inoculating with *Streptococcus haemolyticus* and *Streptococcus viridans* before using. These bacteria always grew without difficulty.

The infected rabbits were either killed when showing typical symptoms, or allowed to die and the approximate time elapsing between autopsy and death noted.

In the first series of experiments an emulsion of the brain of the infected rabbits and the filtrate of this emulsion through a Berkefeld V filter were cultivated in the meat tubes. The tubes were heated to 100°C. and cooled quickly just before inoculation. Each tube was sealed with vaseline and incubated for five to seven days at 37°C. Smears were then made from each tube, also aerobic and anaerobic subcultures.

The results are given in table 1.

In the second series, at Dr. Evans' suggestion, the medium was not heated and cooled just prior to inoculation, and the vaseline seal was omitted. Filtration of the brain emulsion was also suspended.

Results of the second series are given in table 2.

#### PATHOGENICITY OF ISOLATED ORGANISMS

The pathogenicity of the strain of *Streptococcus viridans* isolated by Dr. H. Yu from Dishman virus brain No. 66 was investigated. Two rabbits were injected intracerebrally with 0.25 cc. each of a twenty-four-hour culture of the streptococcus. They showed a febrile reaction in the first forty-eight hours after inoculation, but were otherwise negative. Six weeks afterwards they were tested for immunity against the Dishman virus and both of them died with typical symptoms, thus showing that the previous injection with the streptococcus culture had conferred no immunity against the virus.

Subsequently, four more rabbits and several mice were inoculated intracerebrally with a culture of the same streptococcus. All were negative.

Cultures from four strains of Gram-positive rods and three strains of diphtheroids were also tested by intracerebral inocula-

tion into rabbits. The rabbits were observed for five weeks, but showed no symptoms of encephalitis.

#### SUMMARY AND CONCLUSIONS

In the course of this work cultures were taken from a total of 80 rabbits, which were being used at the same time for other forms of experimentation on herpes virus. The rabbits investigated in the present connection included 5 inoculated with our own strain, 39 with the Perdrau strain, 10 with Evans' Devlin strain and 26 with her Dishman strain. All of these came down with the typical disease except two or three of the first Dishman rabbits which showed slight variation from the ordinary course of the disease. Later, however, this strain corresponded clinically with the others.

After death the brains of these rabbits were removed, emulsified in saline and inoculated into meat tubes. Out of a total of 550 tubes inoculated in this way, a staphylococcus was recovered four times, *B. coli* three times, diphtheroids three times, *M. cremoides* once, Gram-positive rods four times and a streptococcus once. The shorter the interval between death and autopsy, the smaller was the number of organisms recovered from the brain, and it was also noted that with more experience in the technique, fewer organisms were found. It is our belief from these experiments that wherever organisms were recovered, this was due either to non-specific infection of the brain tissue, just before or just after death, accidental contaminations in the course of removal of the brain, and inoculation, or insufficient sterilization of the meat medium. We have found occasionally that the meat medium as prepared for this work was not completely sterilized even by the ordinary method of autoclaving, this probably being the result of insufficient mincing of the meat, with the result that large masses remained in the tubes. In many cases in which we attribute the presence of the organisms to this accident, growth did not appear for as long as seven or eight days.

Rabbits were inoculated with cultures of the organisms iso-



lated, but in no case did encephalitis at all comparable with the herpetic encephalitis of rabbits develop.

We feel justified in concluding that our experiments failed to show any causal relationship between the ordinary visible bacteria and encephalitis and furnished no evidence whatever to support the suggestion that the filtrable encephalitis virus may change into a true bacterial form.

#### REFERENCES

- (1) EVANS, ALICE C., AND FREEMAN, WALTER: Pub. Health Rep., **41**, June 4, 1926.
- (2) EVANS, ALICE C.: Pub. Health Rep., **42**, January 21, 1927.

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